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STUDIES ON ETHIDIUM BROMIDE

V. THE TREATMENT OF CATTLE INFECTED WITH RESISTANT STRAINS OF *Trypanosoma congolense*

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INTRODUCTION

It is well known that strains of *T. congolense* may become resistant to drugs when exposed to sub-curative doses. These resistant strains can often be effectively treated by the administration of greater amounts of the drug to which they are resistant, providing the therapeutic index of the drug allows such higher dosage: they may or may not be eliminated from the blood stream by the standard curative doses of drugs other than the one to which they are resistant.

It was therefore necessary to determine the effect of ethidium bromide on strains of *T. congolense* resistant to 1.5 mg/kg. of dimidium bromide and also to 5.0 mg/kg. of antrycide methyl sulphate, these being the dose levels normally employed for field therapy. A previous experiment in this series by Ford, Wilmshurst and Karib (1953) had shown that a dose of 1.0 mg/kg. of ethidium bromide should be satisfactory for the treatment of *T. congolense* infections and therefore a strain was made resistant to this level of the drug and treated with the standard doses of dimidium and antrycide.

METHODS AND RESULTS

The cattle used were 1½ to 2 year old bulls from the Northern Sudan which were immunised against rinderpest and pleuro-pneumonia, tethered in shaded compounds and fed on burseem and durra stalks.

The method of producing resistance in the strain of *T. congolense* was similar with each drug. In the case of antrycide, for example, a recently isolated strain of the trypanosome was inoculated into six bulls and, when a heavy blood infection was evident, the animals were treated with 0.25 mg/kg. When a definite relapse was obtained, the strain was subinoculated into fresh bulls, to avoid loss of the strain if relapse did not follow retreatment, or if the bull happened to die, and the donor bull was retreated at 0.5 mg/kg. As relapse occurred the retreatment dose was increased by 0.25 mg/kg steps up to 1.0 mg/kg. and thence by 0.5 mg/kg. steps until finally relapse occurred following the administration of 5.0 mg/kg. antrycide methyl sulphate. This strain was then used to infect the experimental bulls and the rise of infection was followed by the

daily examination of early morning wet blood smears from the ear vein. Using the 2/3rd objective about 200 fields were examined.

After treatment, daily blood smears were stained by a modified Fields method (Neave, 1952) and about 200 fields were examined under the 1/12 objective.

Experiment 1. The treatment of T. congolense *resistant to 1.5 mg/kg. dimidium bromide*

Thirty bulls each received a subcutaneous injection of 5 cc. of citrated blood from a bull which had relapsed after treatment with dimidium bromide at a level of 1.5 mg/kg. Fourteen days after the infection all bulls showed a parasitaemia. They were divided into five random groups and treated as detailed in Table 1. Ethidium bromide was administered to three groups. Group I received 1.0 mg/kg. as a 1 per cent solution, group II, 2.0 mg/kg. and group III 5.0 mg/kg. as a 2 per cent solution. Group IV was given dimidium bromide, 1.5 mg/kg. as a 2 per cent solution. Group V were untreated controls. A local swelling was produced at the site of injection and was generally greater in the animals on the higher dose rates. With time, the swellings subsided leaving small skin scars in a few of the animals.

The results of these treatments are given in Table 1. The bloods of all bulls in Groups I to IV became negative within 48 hours of treatment and Groups I, II and III remained free of blood infection during the sixteen week observation period. Group V was included to demonstrate the viability of the infecting strain and one of the bulls in this group died of trypanosomiasis four weeks after infection. Previous experience in the Sudan had shown that dimidium resistant strains of *T. congolense* could be cured by antrycide methyl sulphate and therefore six weeks after they had been infected the remaining five bulls of Group V were given antrycide methyl sulphate at a rate of 5.0 mg/kg. Their bloods remained free of parasites for the remainder of the experiment. Group IV had been included to confirm the resistance of the strain to 1.5 mg/kg. dimidium bromide. There was one death from trypanosomiasis in the second week after treatment; relapses commenced in the third week and by the end of the fourth week all five bulls had relapsed. Two bulls died from trypanosomiasis in the sixth week and the remaining bulls were retreated with ethidium bromide at a rate of 1.5 mg/kg. and their bloods were free of parasites for the remainder of the experiment.

This experiment therefore showed that the treatment of a *T. congolense* infection resistant to dimidium bromide 1.5 mg/kg. with ethidium bromide at levels of 5.0, 2.0 or 1.0 mg/kg. caused the disappearance of trypanosomes from the blood which remained free during a 16 week observation period. Treatment of two animals with 1.5 mg/kg. of ethidium bromide seven weeks after infection

TABLE 1

THE TREATMENT OF *T. congolense* MADE RESISTANT TO DIMIDIUM BROMIDE 1.5 MG/KG.

Group	*Drug*	*Dose mg/kg.*	*No. of bulls*	*Daily blood examinations. Number of bulls showing one or more positive slides during week*															
				Before treatment	*Weeks after treatment*														
					1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
I	Ethidium	1.0	6	6	4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
II	Ethidium	2.0	6	6	4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
III	Ethidium	5.0	6	6	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
IV	Dimidium	1.5	6	6	1 dth 5	—	3	5	5	3	★	—	—	—	—	—	—	—	—	—
V	None	—	6	5	6	1 dth 5 1 dth	5	5	†	2 dths —	—	—	—	—	—	—	—	—	—	—

★One bull was removed and the two remaining were treated with ethidium bromide 1.5 mg/kg.

†The five remaining bulls were treated with antrycide methyl sulphate 5.0 mg/kg.

NB. The positive smears in week 1 in Groups I to IV all occurred in the 24 hours after treatment.
All deaths were due to clinical trypanosomiasis.

TABLE 2

THE TREATMENT OF *T. congolense* MADE RESISTANT TO ANTRYCIDE METHYL SULPHATE 5.0 MG/KG.

Group	Drug	Dose mg/kg.	No. of bulls	Before treatment	Daily blood examinations. Number of bulls showing one or more positive slides during week — Weeks after treatment																								
					1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
I	Ethidium	1.0	6	6	–	1	3	5	6	6	6	6	6	6* 1 dth	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
II	Ethidium	2.0	6	6	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–		observations ended								
III	Ethidium	5.0	6	6	–	–	–	–	–	–	–	–	–	–	–	–	–	1 dth	–	–		observations ended								
IV	Antrycide	5.0	6	6	–	–	–	–	3	4 1 dth	4	4	4	5	5	4	3	3	3	3 1 dth	2	observations ended								
V	None	–	6	6	6	6	6 1 dth	5 1 dth	4	4	1 3 dth	1 1 dth		all	con	trol	s de	ad											

*Remaining 5 bulls retreated with ethidium bromide 5.0 mg/kg.
The death in Group II in week 14 was due to pleuro pneumonia.
All other deaths were due to clinical trypanosomiasis.

also eliminated trypanosomes from the blood which then remained free during the remaining nine weeks of the experiment.

Experiment 2. The treatment of T. congolense *resistant to 5.0 mg/kg. antrycide methyl sulphate*

For this experiment the resistance of a strain of *T. congolense* to antrycide methyl sulphate had been raised to 5.0 mg/kg, and thirty bulls were each injected with 5 cc. of infected blood. Fourteen days after infection, when all the bloods were positive, the bulls were divided into five groups and treated as detailed in Table 2, which also shows the results of treatment. The rise of the infection and results of treatment were observed as described under experiment 1.

The untreated animals of Group V demonstrated the virulence of the strain as, by the tenth week after infection, all six bulls had died from clinical trypanosomiasis.

Group IV demonstrated the antrycide-resistant nature of the strain. It is interesting to note that although two deaths from trypanosomiasis had occurred seventeen weeks after treatment, i.e. nineteen weeks after infection, four bulls remained alive and parasites were infrequent in their blood. It is possible that, compared with Group V, the extra exposure to antrycide 5.0 mg/kg. may have reduced the virulence of the trypanosome.

The results obtained in Groups II and III showed that treatment of the antrycide resistant strain of *T. congolense* with ethidium bromide at 2.0 or 5.0 per mg/kg respectively caused the disappearance of trypanosomes from the blood which remained free for sixteen weeks. A dose of 1.0 mg/kg was insufficient (Group I) but retreatment of these animals with 5.0 mg/kg ethidium bromide confirmed the Group III results.

Experiment 3. The treatment of T. congolense *resistant to 1.0 mg/kg. ethidium bromide*

The resistant strain of *T. congolense* for this experiment was derived from a relapsing animal in the treatment experiment described by Ford, Wilmshurst and Karib (1953). This bull infected with a field strain of *T. congolense* had relapsed five weeks after treatment with ethidium bromide at 0.5 mg/kg. Retreatment carried out at 0.75 mg/kg. was followed three weeks later by a second relapse and a second retreatment at 1.0 mg/kg. was followed by a third relapse in five weeks. Twenty-three bulls were each infected with 5 cc. of citrated blood and, when all were blood positive, treatment was carried out according to table 3. The rise of infection and results of treatment were followed by the same methods as described for experiments 1 and 2.

Group III confirmed the drug resistance and group IV the viability of the strain of *T. congolense*. After five weeks of observations the bulls were subjected to further drug treatments to increase the

TABLE 3

THE TREATMENT OF *T. congolense* MADE RESISTANT TO ETHIDIUM BROMIDE 1.0 MG/KG.

Group	*Drug*	*Dose mg/kg.*	*No. of bulls*	*Daily blood examinations. Number of bulls showing one or more positive slides during week*																					
				Before treatment	*Weeks after treatment*																				
					1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
I	Antrycide	5.0	6	6	–	–	–	–	–	–	–	–	1	1	1	1	1	1†	–	–	–	–	–	–	–	–
II	Dimidium	1.5	6	6	6	6	6	6	6*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	
III	Ethidium	1.0	5	5	5	5	5	5	5				Retreated with increasing doses of ethidium to build up further drug resistance.													
IV	None	–	6	6	6	6	6	6	5 1 dth																	

*Retreated with antrycide methyl sulphate 5.0 mg/kg.

†The one relapsed animal was retreated with ethidium bromide 5.0 mg/kg. and remained free of parasites for a further 16 weeks. Observations on the other five bulls ceased at 16 weeks.

resistance of the trypanosome to ethidium bromide for future experiments.

Group II demonstrated the failure of dimidium bromide 1.5 mg/kg. to overcome resistance to ethidium bromide at 1.0 mg/kg., while retreatment with antrycide methyl sulphate at 5.0 mg/kg. cleared the blood of trypanosomes which did not reappear during sixteen weeks further observations. However, in Group I, treatment by antrycide 5.0 mg/kg. of the ethidium resistant parasite which had not encountered dimidium bromide 1.5 mg/kg. was followed by relapse in one bull nine weeks after treatment. Retreatment of this one animal by ethidium bromide at 5.0 mg/kg. cleared trypanosomes from the blood which remained free for sixteen weeks of observations.

DISCUSSION

When mass field treatments are carried out with trypanocidal drugs there are often ample opportunities for the development of drug fastness, as under-dosage may arise from inaccurate weight estimation, accidents at injection, imperfect preparation of solutions, or from reinfection at a time when the blood level of drug is waning. It is therefore necessary to know what increase in dosage is required to deal with resistance to a particular drug and also whether the normal curative doses of other drugs are active against the resistant strains.

These experiments have confirmed that by artificial means *T. congolense* may be made resistant to the generally accepted curative doses of antrycide and dimidium i.e. 5.0 mg/kg. of antrycide methyl sulphate and 1.5 mg/kg. of dimidium bromide. They have also shown that resistance to ethidium bromide at 1.0 mg/kg. can be produced. If the criterion of cure is the disappearance of trypanosomes from the blood, their absence for a period of sixteen weeks and the maintenance of a satisfactory clinical condition in the bull, it may be inferred that at the drug levels mentioned in this article, dimidium resistance can be overcome by ethidium (experiment 1) but ethidium resistance cannot be treated by dimidium (experiment 3), whilst antrycide resistance is not overcome by ethidium (experiment 2) and ethidium resistance whilst affected by antrycide is not completely overcome (experiment 3).

If, on the other hand, the dose of ethidium is increased to 2.0 mg/kg., as its therapeutic index allows, then antrycide resistance is overcome (experiment 2). In the case of one animal in experiment 3 (Group I) an increase of the dose of ethidium to 5.0 mg/kg. overcame resistance to ethidium 1.0 mg/kg. which antrycide had failed to cure. It will be necessary to carry out further experiments to determine what is the smallest increase over 1.0 mg/kg. in the dose of ethidium necessary to deal with *T. congolense* which has become resistant to either ethidium bromide or antrycide methyl sulphate.

CONCLUSIONS

A strain of *T. congolense* resistant to dimidium bromide at 1.5 mg/kg. was cured by ethidium bromide at 1.0 mg/kg.

A strain of *T. congolense* resistant to antrycide methyl sulphate at 5.0 mg/kg. was cured by ethidium bromide at 2.0 and 5.0 mg/kg.

A strain of *T. congolense* resistant to ethidium at 1.0 mg/kg. was cured by ethidium at 5.0 mg/kg., but, when antrycide at 5.0 mg/kg. was used, there was one relapse out of six animals : this animal was cured by ethidium at 5.0 mg/kg.

For the purpose of this experiment cure means freedom from blood infections during sixteen weeks of daily examination.

ACKNOWLEDGMENTS

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SOME OBSERVATIONS ON THE FREE LIVING STAGES OF THE CATTLE LUNGWORM IN RELATION TO THEIR NATURAL ENVIRONMENT

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INTRODUCTION

A recent field investigation of the epidemiology of parasitic bronchitis in the adult carried out by one of us indicated that success of the larvae of *D. viviparus* in finding their way to the host was in some way influenced, among other factors, by the state of the herbage and perhaps by the consistency of the faeces. Especially it appeared that rapidly growing leys rich in clover might represent an especial danger. Since the trend of recent developments in grassland management appears to be increasingly towards this type of sward, experimentation on the effect on the larvae of factors related to the herbage appeared desirable.

The literature on the bionomics of lungworm larvae is very scanty. Daubney (1920) described the larvae and maintained that under certain conditions the infective larvae of *D. viviparus* will perform a slight vertical migration. This we have been unable to confirm and we are of the opinion that the larvae of *D. viviparus* do not climb up the herbage. Orlov (1946) also came to this conclusion and suggested that the larvae depended on flood water to liberate them from the faecal mass. The latter writer also found that during the summer the larvae do not persist on pastures longer than three weeks. Porter (1941) similarly concluded that in Alabama during May and also in August and September the larvae persisted for more than one week but less than six. Soliman (1952) concluded that the larvae did not persist for two months in winter in Southern England.

There is a much greater volume of published information on the larvae of the trichostrongylidae and in so far as work on lungworm larvae is liable to be based on this, we would suggest that much of this information is of such a nature that its contribution to an understanding of the circumstances relevant to the epidemiology of lungworm disease is severely limited. Thus many investigators are at pains to amass data concerning the reactions of the larvae to different environmental factors, to isolate one factor from another and, above all, to make accurate measurements of the limits of tolerance of the larvae. Information of this type is of very limited value since the quantities which can be measured in the laboratory can not be measured nor even estimated in the microclimate to which the larvae may be exposed in the field.

The epidemiologist wishes to know the effect of different factors on the numbers of larvae available to the grazing animal and it is not essential to understand every step in each train of causation in order to acquire a knowledge of its effects.

The concept of availability to the grazing animal is complex. In order to be available to the grazing animal the larva must develop to the infective stage, it must survive and it must be so situated in relation to faeces and herbage that the grazing animal is liable to swallow it; and this last is dependent also on the grazing behaviour of the animal. Availability to the host, since one of its components depends on grazing behaviour, is a difficult quantity to measure. Indeed it can only be investigated by means of grazing experiments so that even a modest assessment of the effects of only a few factors would constitute a major enterprise requiring large areas and surprisingly large numbers of calves. While it is necessary to observe something less than the entire process it it unnecessary and indeed undesirable to swing to the opposite extreme and study the properties of the larva out of relation to its normal environment.

The present paper reports the beginnings of a study of the larvae of *D. viviparus* in their natural environment and the influence on their development, survival and situation of a number of simple factors which appear to be relevant to the effects of different husbandry practices. It also draws attention to the nature of the problem and to what we believe to be a direct and relevant approach to it and one which might with profit be employed on related problems.

METHODS

The first observations were made on pats of faeces placed out of doors on sward which had first been cut to a uniform height of two inches. Subsequent experiments were performed with infected faeces placed on rapidly growing grasses in boxes. The boxes were constructed of galvanised iron and measured 18 in. square x 8 in. high. Holes in the bottom allowed for drainage which was further facilitated by a layer of broken pot. Above this was a layer of loam upon which turves were laid. These turves were cut from a clover ley. The predominant species were S23 rye grass and S100 white clover and care was taken in selecting the turves to ensure that grasses and clovers were uniformly distributed. Long grass was compared with short. This "short" grass was cut to a length of two inches at the start of each experiment and was thereafter allowed to grow. In consequence it was materially shorter than the "long" grass only for the first three weeks.

The boxes were kept in cold frames to protect them from rain. Some were watered from below, being given three pints of water daily from a a watering can fitted with a rose. Others which were watered from above, stood in shallow metal trays kept filled with water. Faeces were put on the boxes either as an undisturbed pat or were thinly spread over the herbage. The "undisturbed" faecal pat took the form of a central strip six inches wide right across the box. Four pounds of faeces were used which gave a pat of an average thickness of $1\frac{1}{2}$ in. The "spread" faeces weighed 1 lb. per box and were, after thorough mixing, uniformly spread with a spatula over the herbage of the entire box. In calculating percentage recoveries the difference in the weight of faeces used was allowed for.

Each experimental treatment was replicated on several boxes. At weekly intervals random samples were taken from one of the replicates. The box was divided into quarters out of which all the grass (save a $\frac{1}{2}$" strip at the edges of the box) and a ten gram sample of faeces were taken. The surface of the soil was scraped to remove a layer approximately $\frac{1}{4}$ in. thick. This was also examined for the presence of larvae.

The procedures used for the recovery of larvae from faeces and soil and from herbage embody no new principles but, since the third stage larvae of *D. viviparus* are inactive, the actual separation is accomplished by flotation and not by means of the Baermann apparatus as used in Taylor's method for the recovery of trichostrongylid larvae from herbage.

The recovery of larvae from faeces or soil. A ten gram sample of faeces is shaken with about 70 cc. of water in a wide mouthed bottle containing about 40 glass balls of $\frac{1}{4}$ in. diameter, until the faeces are entirely broken down and an even suspension is obtained. From this the larger particles are removed by means of a wire screen having 80 meshes to the linear inch. The suspension is encouraged to pass through the screen by playing on it a forceful jet of water from the tap. The fluid that has passed through the screen (about 500 cc.) is allowed to stand in a round bottomed glass vessel undisturbed for some three hours. The supernatant fluid is syphoned off and the sediment is transferred to four Clayton Lane centrifuge tubes which are spun for two minutes at 2,000 r.p.m. The sediment which has now been freed of both the very large and the very small particles is resuspended in saturated salt solution. The tubes are centrifuged again and a little salt solution is added to each to make a positive meniscus. A small glass square is placed on the top of each tube and they are spun for two minutes at 1,200 r.p.m. The glass squares are lifted off and the larvae are washed into a watchglass. Where the numbers are reasonably small the contents of the watchglass are further centrifuged in a conical tube and the sediment transferred to a slide for microscopic examination. If inconveniently large numbers are present the contents of the watchglass are made up to 50 cc. and three samples each of 5 cc. are examined.

The recovery of larvae from the herbage. The herbage is cut at soil level and having been weighed is cut up into pieces not more than 2 in. long. It is placed in four-litre pneumatic troughs, not more than 100 g. to a trough, and a fine jet of water is played upon it until the trough is three parts full. The contents are stirred several times during the day and allowed to stand overnight. The herbage is removed and the water squeezed out with the hands. The fluid is allowed to stand another night and most of the supernatant fluid is then syphoned off. The residue, perhaps 500 cc. is poured through a coarse sieve to remove small pieces of grass and transferred to a round bottomed glass vessel. From this point a procedure identical with that already described for the enumeration of larvae in soil or faeces is employed. The herbage is subjected to the washing process three times and all three washings are counted separately. Though a proportion of the larvae is frequently found in the second washings, their appearance in the third is rare.

RESULTS

Observations on Faeces out of Doors

Freshly passed pats of infected faeces placed on pasture land were examined at weekly intervals. Two ten g. samples were taken,

one from the centre of the pat, the other from the edge and the number of live larvae determined. These pairs of figures did not reveal any constant relationships between the concentration of larvae in the two sites. Their means shown in Table 1 are also expressed as a percentage of the number originally present. Twenty g. samples of herbage were also taken weekly. Earlier observations had shown that very few larvae could be found more than two inches away from the faeces and in consequence the herbage taken for these observations was cut within this radius (Table 1). As a rule a new pat of infected faeces was put out as soon as no more larvae were recoverable from the faeces or herbage under observation.

TABLE 1

SURVIVAL OF LARVAE IN FAECES AND ON HERBAGE OUT OF DOORS

Date	*Week*	*Number of larvae in 20 g. faeces*	*Percentage in Faeces*	*Number of larvae in 20 g. herbage*
October 24th	0	1650	100	0
	1	1041	63.09	0
	2	422	25.58	4
	3	215	13.03	19
	4	55	3.33	6
	5	50	3.03	2
	6	35	2.12	0
	7	10	0.61	0
	8	21	1.27	2
	10	3	0.18	1
	11	0	0	0
	12	2	0.12	1
	13	2	0.12	0
	14	0	0	0
	15	0	0	0
	16	0	0	0
February 6th	0	2046	100	0
	1	1325	64.8	32
	2	420	20.5	15
	3	63	3.08	9
	4	16	0.78	4
	5	3	0.15	1
	6	0	0	1
	7	0	0	0
March 19th	0	1968	100	0
	1	192	9.8	48
	2	48	2.4	16
	3	16	0.81	4
	4	0	0	4
	5	0	0	0
April 2nd	0	2184	100	0
	1	1176	53.9	32
	2	304	13.9	16
	3	56	2.6	4
	4	4	0.18	6
	5	0	0	0
	6	0	0	0

TABLE 1—*contd.*

Date	*Week*	*Number of larvae in 20 g. faeces*	*Percentage in faeces*	*Number of larvae in 20 g. gerbage*
May 14th	0	1580	100	0
	1	160	10.1	30
	2	30	1.9	20
	3	10	0.63	15
	4	0	0	0
	5	0	0	0
June 11th	0	2470	100	0
	1	899	36.4	0
	2	41	1.66	4
	3	11	0.45	13
	4	0	0	15
	5	0	0	20
	6			17
	7			12
	8			0
	9			1
	10			0
	11			0
July 9th	0	1364	100	0
	1	1248	91.5	0
	2	25	1.8	7
	3	11	0.81	5
	4	4	0.29	2
	5	0	0	0
	6	0	0	0
July 30th	0	2225	100	0
	1	1245	56	8
	2	223	10	12
	3	3	0.13	37
	4	0	0	2
	5	0	0	0
	6			1
	7			0
	8			
August 27th	0	2025	100	0
	1	1525	75·3	30
	2	399	19·7	10
	3	71	3·5	1
	4	8	0.39	2
	5	2	0.10	2
	6	3	0.15	1
	7	1	0.05	1
	8	1	0.05	0
	9	0	0	0
	10	0	0	

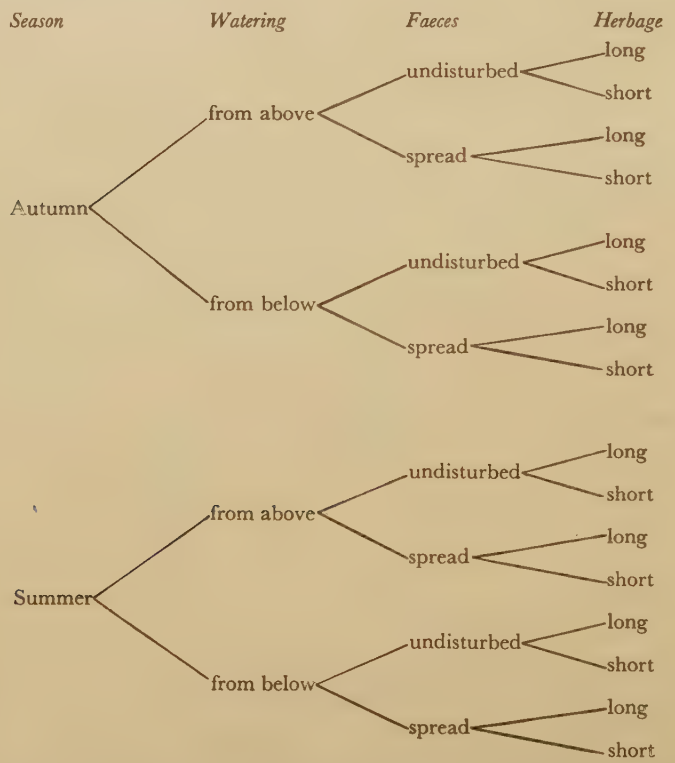
It will be seen that the numbers on the herbage even quite close to the faeces represents only a very small proportion of those on the faeces. Perhaps an even more remarkable phenomenon is the great initial mortality. At least 90 per cent of the larvae died in the first three weeks, and generally in the first fortnight. The subsequent survival of the remainder depended on the season and was found

to be longer in autumn and early winter than in spring and summer, the total period of survival varying from three weeks in May to thirteen weeks in the period October to December.

The Experimental Assessment of the Effect of Various Environmental Factors

It appeared from the outdoor observations that the migration of larvae from the faecal pat is so limited that other factors must operate in the field to make the larvae accessible to the grazing animal and it may well be that it is the faeces themselves rather than the larvae that get on to the herbage by the agency of animals' feet or when the faeces are diarrhoeic. It was decided to compare the survival of larvae in thin layers of faeces on the herbage that might result from such spreading with that in the undisturbed faecal pat and the grass surrounding it. At the same time the effect of three other factors, the length of the herbage, the presence or absence of rain and the time of year was also studied.

This experiment was carried out on faeces placed on the grass boxes already described. The full lay-out of the experiment was as follows :—



Since four factors in all were investigated 16 treatments resulted.

The observations on undisturbed faeces in boxes confirmed the findings made with faeces out of doors. There was a heavy initial

mortality as is seen in Table 2. Very few larvae succeeded in reaching even the herbage adjacent to the faeces and the numbers recovered from herbage more than 2½ inches away from the faeces were almost negligible. This appears from Table 3 in which the larvae recovered are shown as a percentage of those originally present in the faeces.

TABLE 2

PERCENTAGE SURVIVAL OF LARVAE IN THE UNDISTURBED FAECAL PAT

Week	*Autumn*				*Summer*			
	Watered from below		*Watered from above*		*Watered from below*		*Watered from above*	
	Long herbage	*Short herbage*	*Long herbage*	*Short herbage*	*Long herbage*	*Short herbage*	*Long herbage*	*Short herbage*
1	5.0	1.9	46.6	40.2	9.1	13.7	13.1	6.8
2	18.9	6.7	40.7	3.3	4.6	5.1	6.8	2.9
3	15.1	7.4	3.3	0.3	3.4	2.3	1.7	2.3
4	1.0	3.9	—	1.4	1.1	1.1	1.1	2.9
5	7.7	0.1	0.8	0.8	0.6	2.9	—	0.6
6	18.0	0.3	0.5	0.5	2.3	1.7	—	—
7	8.4	0.2	—	0.5	—	0.6	—	—
8	8.6	0.1	0.3	0.3	—	0.6	—	—
9	1.2	—	—	2.4	—	—	—	—
10	—	—	—	—	—	—	—	—
12	1.2	—	0.3	—	—	—	—	—
14	—	—	0.3	—	—	—	—	—
16	—	—	—	—	—	—	—	—

Table 4 shows the numbers of larvae recovered from the herbage also expressed as a percentage of the original number in the faeces. It will be seen that in the case of the spread faeces the usual heavy initial mortality occurred. In all cases the numbers recoverable from the herbage in the "spread" boxes was substantially greater than in the undisturbed, especially where the herbage was long. However, while the numbers on the herbage were greater where the faeces had been spread, the survival in the spread faeces was not as great as in undisturbed faeces (Table 2 and 4) but it must be considered that it is only when the larvae are on the herbage that they are epidemiologically significant.

The length of the herbage had only a limited effect on survival of larvae in undisturbed faeces but had a very marked effect on the survival of those on the herbage. When watering was from below, the number recovered from the long herbage greatly exceeded those on the short, especially in autumn. When watering was from above the difference between long and short herbage was less, a result which would tend to confirm the supposition that the length of the herbage acts through the humidity of the microclimate amongst it. The percentage of larvae recoverable from the herbage where watering was from above was somewhat less than where watering

TABLE 3

MIGRATION OF LARVAE FROM UNDISTURBED FAECAL PAT

Living larvae recovered from the herbage expressed as a percentage of the number originally present in the faeces.

	Watered from below							
	Autumn				*Summer*			
	Long herbage		*Short herbage*		*Long herbage*		*Short herbage*	
Week	*<2½″ from faeces*	*>2½″ from faeces*	*<2½″ from faeces*	*>2½″ from faeces*	*<2½″ from faeces*	*>2½″ from faeces*	*<2½″ from faeces*	*>2½″ from faeces*
1	.107	.003	.040	.001	.813	.038	1.050	0
2	.392	.021	.013	.021	.248	.013	.200	.088
3	.286	.045	.016	.006	.125	.063	.150	.013
4	.021	0	.082	.003	.050	0	.013	.013
5	.165	.004	.016	0	0	0	.025	0
6	.385	.010	.006	.001	0	0	0	0
7	.172	.013	.004	0	0	0	0	0
8	.188	.001	.001	0	0	0	0	0
9	.026	0	0	0				
10	0	0	0	0				
12	.027	0	0	0				
14	0	0	0	0				
16	0	0	0	0				
	Watered from above							
1	.014	0	.010	0	.500	0	.188	.038
2	.001	.001	.011	.004	.750	.113	.875	.188
3	.002	0	0	0	.175	.025	.225	.036
4	.005	.001	0	0	.138	.038	.150	.038
5	0	0	.002	.001	.013	0	.013	0
6	.004	0	.001	0	.025	0	.013	0
7	.001	0	0	0	0	0	0	0
8	.001	0	0	0	0	0	0	0
9	.003	0	0	0	0			
10	0	0	0	0	0			
12	0	0	0	0				
14	0	0	0	0				
16	0	0	0	0				

was from below. This is not due to the larvae being washed off the herbage since the numbers recovered from the soil were negligible and did not differ significantly as between the boxes watered from above and from below. We would suggest that the difference is explicable in terms of the laboratory observation that alternate wetting and drying is more harmful to the larvae than gentle drying. It would also appear that rain does not facilitate the migration of larvae out of the faeces (Table 3).

As found in the observations out of doors, survival in summer was less than in autumn and the variation between long and short

TABLE 4

COMPARISON OF SPREAD AND UNDISTURBED FAECES

Living larvae recovered from the herbage expressed as a percentage of the number originally present in the faeces.

Week	*Watered from below*							
	Autumn				*Summer*			
	Spread faeces		*Undisturbed faeces*		*Spread faeces*		*Undisturbed faeces*	
	Long	*Short*	*Long*	*Short*	*Long*	*Short*	*Long*	*Short*
1	46.88	1.21	0.11	0.24	3.85	1.15	0.85	1.05
2	12.77	0.34	0.41	0.15	0.85	0.10	0.25	0.30
3	6.96	0.40	0.33	0.16	0.70	0	0.19	0.16
4	0.26	0.11	0.02	0.09	0.70	0.02	0.05	0.03
5	0.10	0.02	0.17	0.001	0.15	0	0	0.03
6	0.38	0.01	0.40	0.007	0.05	0	0	0
7	0.22	0.01	0.18	0.004	0	0	0	0
8	0.05	0.01	0.19	0.001	0	0	0	0
9	0.09	0.01	0.03	0				
10	0.05	0.015	0	0				
12	0.03	0.005	0.02	0				
14	0.01	0	0	0				
16	0	0	0	0				
	Watered from above							
1	8.83	5.93	0.14	0.10	1.75	1.35	0.5	0.23
2	0.95	0.33	0.02	0.08	0.55	0.05	0.86	0.99
3	0.50	0.05	0.02	0	0.2	0	0.33	0.26
4	0.62	0	0.05	0	0.25	0.1	0.18	0.19
5	0.10	0.05	0	0.02	0.20	0.05	0.01	0.01
6	0.12	0	0.04	0.01	0	0	0.03	0.01
7	0.71	0.02	0.006	0	0	0	0	0.01
8	0	0.02	0.006	0	0	0	0	0
9	0.02	0	0.03	0				
10	0.24	0	0	0				
12	0	0	0.006	0				
14	0	0	0	0				
16	0	0	0	0				

herbage was less marked in summer. Presumably the local humidity amongst the herbage is lower at this time of year and the seasonal incidence of parasitic bronchitis may be associated with the humidity and relative warmth prevailing in autumn.

DISCUSSION

The results here presented have a number of implications relevant to the epidemiology of husk. Only a minority of the larvae survive for any length of time and very small numbers indeed succeed in leaving the undisturbed faecal pat and getting on to the herbage. The number migrating sufficiently far from the faeces to have a reasonable chance of being swallowed by a bovine is negligible. The

conclusion is therefore inescapable that the process in the field must be in some way different and indeed many factors operate which spread the faeces themselves over the herbage. Of these factors spreading by the agency of animals feet is probably important as is the situation when animals are diarrhoeic and their faeces are thinly scattered over a wide area. It may also be that under certain circumstance the use of the chain harrow may be harmful. The thin layers of faeces on the herbage are apparently more sensitive to desiccation than is the undisturbed faecal pat, but when the microclimate amongst the herbage is humid as it will be in tall dense herbage, especially if it is rich in clover, then faeces spread over the herbage would appear to represent the ideal means of getting large numbers of infective larvae onto the herbage.

Before larvae on the herbage can be epidemiologically meaningful, however, they must be so situated as to be available to the grazing animal. Little is known of the grazing behaviour of bovines in regard to the contamination of herbage with bovine faeces, but it is true to say that the bovine will avoid grazing near obvious accumulations of such faeces. In the case of the undisturbed faecal pat the larvae that matter are those at some distance from the faeces and as we have seen these are negligible. We would suggest that when the faeces are very thinly spread the grazing bovine is not able to avoid the contaminated herbage.

It appears, therefore, that the undisturbed faecal pat from which less than two-tenths of one per cent of the larvae migrate to a point where they are meaningful is in marked contrast to the spread faeces which in our experiments has made available nearly fifty per cent of the larvae after one week or seven per cent after three weeks. This circumstance would tend to support the indication obtained from field observations that the actual number of larvae being passed on to the ground in the faeces is less important in determining the numbers available to the grazing animal than are a number of other factors of which the state of the herbage and the consistency of the faeces are two.

The experiments reported give some indication of what general conditions are favourable to the process of infection. High humidity and not too low a temperature will favour the larvae but showery weather with bright intervals is presumably harmful, as is drought and drying by sun and wind. This impression is not contradicted by observations that have been made on the level of infestation on the herbage of experimental paddocks which indicated that the weather may have a very marked influence on the numbers of larvae present. It is not possible at this stage to define precisely what climatic conditions are the most favourable to the larvae. It does appear, however, both from these observations and the experiments described in the present paper, that conditions favouring availability and survival of the larvae are more liable to occur in autumn than at any other season and it is felt that this must go

some way towards explaining the seasonal incidence of the disease. Of course the climate that is particularly of importance in this context is the local microclimate amongst the herbage and it has been our experience that outbreaks of husk which occur in late spring and summer are often associated with long dense shade-producing herbage, which, as we have seen in these experiments represent another factor favourable to the lungworm larvae especially if its composition is such as to induce animals to scour. Clearly, rapidly growing leys rich in clover provide these conditions and it may be that the great extension in alternate husbandry and in intensive grassland management may have some influence on the incidence and severity of parasitic bronchitis.

SUMMARY

Observations on pats of faeces out of doors and on the surrounding herbage indicated that the period of survival varied from season to season, though the initial mortality was heavy throughout the year. In May the larvae survived for only 4 weeks but they persisted for 13 weeks from October to January.

The majority of the surviving larvae remained in the faeces and did not migrate on to the herbage.

Experiments with infected faeces placed on rapidly growing grasses in boxes showed that survival in autumn was longer than in early summer.

The largest number of infective larvae is recoverable from the herbage when the faeces have been thinly spread upon it. When the herbage is long the initial survival in these thin films of faeces is favoured, especially in autumn.

The longevity of larvae within an undisturbed pat of faeces is influenced to only a limited degree by the character of the surrounding herbage. On the other hand the longevity of the small numbers of larvae which have migrated out of the faeces is markedly greater in long than in short herbage.

It is concluded that for larvae to get onto the herbage in meaningful numbers it is necessary for the faeces themselves to get on to the herbage.

The relevance of these findings to the process of infection and the epidemiology of husk is discussed.

ACKNOWLEDGMENT

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THE INCREASED RESISTANCE OF THE UDDER OF GOATS TO INFECTION WITH *STR. DYSGALACTIAE* STRAIN 419, AFTER VACCINATION WITH THE SAME STRAIN

By

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INTRODUCTION

Streptococcus dysgalactiae (Lancefield group C) is recognised as one cause of bovine mastitis. Using strain 419 of this organism, Holman, Smith and Pattison (1953) were able to produce an experimental mastitis in goats. This condition was similar to the experimental disease in goats produced by *Str. agalactiae*, against which Holman, Pattison and Gordon (1952) had shown that some protection of the udder was possible by the use of a homologous vaccine. After it had been shown that active immunity against *Str. dysgalactiae* could be produced in mice (Smith, 1954), the following experiment was designed to discover if the udder of goats could also be given protection against *Str. dysgalactiae*. The experiment was regarded as a preliminary to immunity experiments in cows.

MATERIALS AND METHODS

Experimental animals. Crossbred goats, each in the early stage of their first lactation, were used.

Organism. Str. dysgalactiae strain 419, whose general characters have been described by Holman, *et al*, (1953), was used.

Test dose. One ml. of a 20-hour Todd-Hewitt broth culture containing approximately 10^9 viable organisms, made up to 10 ml. with Todd-Hewitt broth, was injected through a round-ended hypodermic needle into the teat sinus of one side of the udder.

Vaccine. The vaccine was a 0·3 per cent formolised 24-hour Todd-Hewitt broth culture of *Str. dysgalactiae* strain 419. Prepared freshly each week it was tested for purity and non-viability by plating out on Bactotryptose ox-blood agar plates. Smith had found this vaccine effective in protecting mice. Living *Str. dysgalactiae* could not be used for vaccination of goats because it may cause lameness (Stableforth, 1932; Smith, 1954).

Bacterial counts on milk samples. 0·02 ml .of foremilk was spread on the surface of a Bactotryptose ox-blood agar plate, and colonies were counted after 24 hours incubation. Actual counts were made up to about 200 colonies; between 200 and 500 counts were approximated to the nearest 50; non-confluent counts greater than 500 were recorded as 750, and confluent growth as 2000.

Neutrophil counts on milk samples. Neutrophil counts per ml. of well shaken milk were calculated from counts on 0.02 ml. of milk spread over 1 sq. cm. of a slide.

Serological tests. Plate agglutination tests were carried out weekly on blood serum and on whey from samples obtained from each side of the udder, using a dense antigen of strain 419 (Holman, *et al.*, 1953).

Random samples of recovered strains were identified by the group precipitation method (Lancefield, 1933).

Estimation of milk loss. Milk yield recording began seven days prior to the first vaccination. After inspection had shown that yields remained fairly steady over a period of weeks the yield for each half of the udder for the seven days prior to the intramammary challenge was averaged to give the 100 per cent figure for the daily yield of that gland, and subsequent daily yields were compared with this figure.

Plan of Experiment

Vaccination. The goats were divided into two groups each with five animals. One group was given 10 ml. of vaccine intraperitoneally each week for four weeks and then rested for three weeks. Two more weekly doses were given and, after a second rest period of three weeks, the test dose was injected. The other group received injections of 10 ml. of formolised Todd-Hewitt broth intraperitoneally at the same times.

Recordings. Three pre-infection milk samples were examined for bacterial and neutrophil counts. After challenge, bacterial counts were made twice daily for three days and then once a day until the 24th day, and neutrophil counts were made daily throughout this period. The temperature of each goat was recorded twice daily beginning one week before challenge. Agglutination tests were carried out each week and the antigen used showed one goat in each group to have a titre of 1: 1 before vaccination began.

RESULTS

Clinical

The first and second doses of vaccine caused a malaise lasting about 48 hours in each goat, but it was progressively milder after each dose so that the final dose produced little effect. After the third vaccination goat No. 3 (Tables 1 and 2, and Fig. 2) aborted. The control goats receiving formolised broth were unaffected by the injections.

After intramammary challenge all goats were dull and showed high temperatures about six hours after injection, but most had recovered by the following morning. Other than in the occurrence of crises there was no marked difference in the clinical reaction of the two groups.

Crises, as already described for strain 419 (Holman, *et al.*,1953), were recorded on nine occasions in the control group and occurred in four of the five goats. On one occasion a crisis occurred in a vaccinated goat and produced a transient depression of milk yield.

Milk Yield

The result for each group is shown in Fig. 1 and for individual goats in Fig. 2.

FIGURE 1

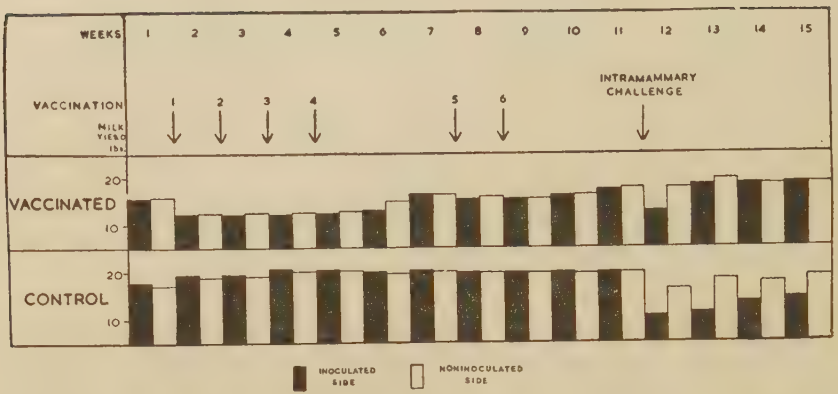

Fig. 1. The effect of six 10 ml. intraperitoneal injections followed by an intramammary challenge of strain 419 on the combined milk yield of
(*a*) five goats receiving injections of a formolised vaccine of strain 419, and
(*b*) five goats receiving injections of formolised broth.

Fig. 1 shows the average drop in milk yield due to the first series of vaccinations in clear contrast to the control group; the milk yield of the vaccinated group recovered during the rest period and sub-

FIGURE 2

Fig. 2. Results averaged for each period of four days showing effects of an injection of 10^9 living strain 419 organisms into the teat sinus of
(*a*) five goats given an homologous vaccine and
(*b*) five goats given formolised broth.

sequent vaccinations caused little loss. After intramammary challenge the greater milk loss of the control group is evident. Although there is an obvious difference between averages in Fig. 1, the differences between individuals show great variation in the

control group. This is due to the facts, as recorded previously by Holman, *et al.*, (1953), that reactions in goats vary irrespective of the size of the intramammary test dose, and that goats giving a minimal reaction can be expected to occur in every group. Holman, *et al.* (1952), faced with the same difficulty in vaccination experiments, chose the second week after challenge as the most suitable

TABLE 1

MILK YIELD PERCENTAGES

Given by the inoculated side of the udder during the second week following intramammary challenge with strain 419 for (a) five goats given an homologous vaccine and (b) five goats given formolised broth.

Day of experiment after challenge	*Vaccinated Goats*					*Control Goats*				
	1	2	3	4	5	6	7	8	9	10
+8	77	104	100	104	122	92	94	0	44	95
+9	77	100	104	93	122	79	85	0	36	86
+10	77	100	100	110	119	104	91	0	33	89
+11	86	122	122	117	122	92	88	0	28	100
+12	86	114	104	117	116	104	82	0	33	78
+13	86	104	100	104	113	92	91	0	39	71
+14	77	118	95	104	106	100	88	0	42	75
Average	80	108	103	107	117	94	88	0	36	84

TABLE 2

MILK YIELD PERCENTAGES

Given by the non-infected side of the udder during the second week following intramammary challenge with strain 419 for (a) five goats given an homologous vaccine and (b) five goats given formolised broth.

Day of experiment after challenge	*Vaccinated Goats*					*Control Goats*				
	1	2	3	4	5	6	7	8	9	10
+8	104	118	110	107	115	96	94	100	88	104
+9	100	118	110	100	115	96	88	84	85	93
+10	95	114	110	110	112	96	91	84	82	93
+11	104	122	119	120	115	100	94	90	88	100
+12	100	114	110	110	112	104	82	95	100	96
+13	104	114	110	110	109	96	91	79	91	93
+14	91	109	100	104	97	89	85	84	85	86
Average	99	115	109	108	110	96	89	88	88	95

time for inspection of the milk yield, and, hence, we have selected this period for further examination; the results are detailed in Tables 1 and 2.

Table 1 records the milk yields of the infected sides of the udder for the second week after challenge. It can be seen that there is great variation in the control group and that the yield for three control goats was greater than that for one vaccinated goat. If, however, 100 per cent is taken as a dividing line the result was as follows :—

Group	*Above* 100%	*Below* 100%	Exact test
Vaccinated . .	4	1	p = 0.0476
Control . .	0	5	

Table 2 records the milk yields for the same period of the non-infected side of the udder, and shows that in each vaccinated goat the average was above the 98 per cent line, in contrast to the control goats that all had averages below this figure. Such a favourable result would be expected to occur only once in 250 times by chance alone.

Neutrophil Counts

These are recorded in Fig. 2 from which it can be seen that milk from control animals contained considerably larger numbers than milk from vaccinated animals. There was, however, overlapping between the two groups.

Recovery of Str. dysgalactiae

Fig. 2 shows that strain 419 was recovered in larger numbers from the milk samples of the control group, although here there was even more overlapping between the two groups than with the other measurements, and the difference between groups is not statistically significant. Thirty-five random samples of recovered strains were identified as *Str. dysgalactiae.*

Antibody Production

With the antigen used, the sera of the vaccinated goats showed titres of about 1 : 4 a week after the first dose of vaccine. After the second dose it rose to 1 : 16 or 1 : 32, and at the end of the course it varied from 1 : 32 to 1 : 128. Following intramammary challenge titres retained their levels in three goats and in the other two fell one dilution to 1 : 16 or 1 : 32. Whey titres were not found until after challenge. They reached 1 : 8 in the whey of the infected side

of the udder in one vaccinated goat three and a half weeks after challenge; lower titres were present in two other goats and the other two remained negative.

In the control goats, low titres were found about fourteen days after challenge. The highest titre recorded was 1 : 4 in both serum and whey, from the infected side of the udder, of goat No. 9, 26 days after infection.

DISCUSSION

The uncertainty as to whether control goats challenged with either *Str. dysgalactiae* or *Str. agalactiae* will react as maximal or minimal reactors, makes precise interpretation of experiments on protection by vaccination difficult. If it were intended to produce a vaccine to give significant protection to the udder more extensive experiments would be necessary to limit the part played by chance. The object of the experiment described here was to find if the use of a vaccine appeared beneficial before carrying out immunity tests in cows, and it is believed that the results give sufficient evidence to warrant such experiments.

The measurement of the estimated loss of milk in the non-infected side following challenge is of interest in that it has given the most statistically significant finding, although it is a measurement we have previously ignored. Indeed in experiments using *Str. agalactiae*, the yield of the non-infected side was used as a basis for the estimation of the loss of the infected side. The meaning of this loss of yield in the non-infected side is not clear but suggests a clinically inapparent disturbance in all non-vaccinated goats, whether minimal or maximal reactors to the test dose. It is known that a crisis produces a slight transient reduction of milk yield of the non-infected side as well as of the infected side and thus it is possible that the greater number of crises occurring in the control group may have produced the result.

SUMMARY

Five of ten goats, in approximately the same stage of lactation, were given a series of six intraperitoneal injections of a formalised vaccine of *Str. dysgalactiae* strain 419; the other five received injections of formalised broth at the same time.

Following intramammary injection of strain 419 into one side of the udder, the vaccinated group showed a smaller loss of milk yield, with fewer neutrophils and bacteria in milk samples, and crises were recorded nine times in the control group and only once in the vaccinated group.

Measurement of the milk yield from non-infected sides of the udder during the second week after the intramammary challenge showed that all the vaccinated goats gave a higher percentage yield

than the controls, although no such group differentiation had been possible before injection of the test dose.

ACKNOWLEDGMENT

We are grateful to Mr. F. H. Summerfield for preparing the photographs.

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MICROGLIAL REACTION IN RABIC INFECTION OF THE MOUSE AND RABBIT

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INTRODUCTION

Soon after its discovery microglia was found to react strongly in established rabic encephalitis (Collado, 1921; Hortega, 1932). Investigating the invasion of the nervous system of the rabbit following inoculation upon the cornea, Field (1954) noted early alteration of the microglia at a stage when the Nissl picture of the nerve cells appeared normal or only minimally affected. Further investigation has shown that such findings are in marked contrast to those in the mouse. Since these results are not without interest from the point of view of function of the microglia they have been recorded here.

MATERIALS AND METHODS

Twenty-five young rabbits have been inoculated upon the cornea by scarification with fixed rabies virus (Pasteur strain kindly supplied by Professor Lepine). Twenty mice were inoculated intracerebrally with 0.02 ml. of 10^{-1} rabic brain suspension, spun for two minutes at 3,000 revs. per minute. The majority of the mice were examined in the later stages of the disease (7 to 9 days). A further twelve mice were subjected to stab wounds of the brain by means of a cold sterile needle and used as controls. Histological examination of the brain was carried out by Hortega's method and by Giemsa staining of paraffin sections.

RESULTS

A detailed account of the development of the infection in the rabbit after corneal inoculation has been given elsewhere (Field, 1954). Even at as early a stage as the seventh day—twenty-four hours after the first occurrence of fever and before the onset of neurological disturbance—a definite alteration of the microglia of the cerebellar (Fig. 1) and cerebral (Fig. 2) cortices was present. Changes in the silver and Nissl pictures are practically contemporaneous. Occasionally, however, the former may show changes more clearly. Thus in E.512—the only animal in which a purulent infection of the inoculated cornea occurred—the disease evolved slowly, and after 24 hours fever which began on the ninth day, nerve cells in the pons were practically normal (Fig. 3A) showing, perhaps, a slight degree of Nissl clumping, whilst microglia was distinctly altered (Fig. 3B).

Later in the evolution of the disease microglial changes become more marked until in the terminal stages at the ninth to eleventh day these cells are largely of "lamellar" type (Collado, 1921), i.e.

they have become converted into flattened triangular or multipolar cells with granular cytoplasm passing off into several short irregular processes which show few branches and none of the hair-like offshoots of non-reacting microglia. Such cells are often to be seen extended along the walls of capillaries, but show no particular disposition to apply themselves to damaged nerve cells (Fig. 4). Evolution beyond this stage to that of compound granular corpuscle formation does not occur in rabies.

In the mouse, on the other hand, generalized microglial reaction has been conspicuously absent even in the last stages of the disease at the seventh to ninth day after intracerebral inoculation. In the neighbourhood of the puncture wound the reaction was severe (Fig. 5) but identical with that seen in the control stab wound of similar duration. The standard Hortega technique used did not stain non-reacting mouse microglia adequately but these cells were observed to acquire full argyrophilic properties and then show structural changes as one approached the wound from the periphery. In the cerebellum no trace of microglial processes was found—a striking contrast from the cerebellar findings in the rabbit. A similar failure to impregnate normal microglia has been recorded by Galego (1928) in cases of canine distemper.

DISCUSSION

The unexpected difference in microglial reaction in the rabbit and the mouse in the face of rabic infection underlines the caution with which general conclusions must be drawn from experiments on a single species. In the present series both animals had approximately the same period of exposure to the virus, the mouse, if anything, a little longer because of the direct intracerebral route, and clinically they presented disturbances of similar order of severity.

These findings raise the question of the function of microglia. This element is usually regarded as the cerebral representative of the histiocytic system of the body concerned with the removal of disintegrated nervous tissue. Hortega (1932) has summed up the general belief that microglia ". . . undergoes modifications during the following processes : (a) whenever there is local disintegration of the nervous tissue and when the nerve and neuroglia cells experience important degenerative disturbances; (b) during intense inflammatory conditions; (c) in the course of hemorrhagic processes. The materials or elements that attract the microgliocytes are, therefore, neurone debris or the products of abnormal trophic metabolism of the neurones (neurophagia), erythrocytes and their remnants (hemophagia), leucocytes and dead cells (cytophagia)." No inflammatory process is, however, visible in rabbit rabies when microglial changes make their appearance.

The relation between nerve cell damage and microglial reaction has engaged the attention of a number of workers. On the one hand, Meduna (1928) claimed that a certain independence of

histologically demonstrable reaction existed, and the same view has been expressed by Spielmeyer (quoted by Creutzfeltdt and Metz, 1926). These latter authors feel that there is a general parallelism between nerve cell sickness and microglial alterations. Whilst the present work suggests that the latter may precede the former, this dissociation may well result from insensitivity of our histological criteria of nerve cell damage.

Meduna (1927) has suggested that microglia, by its ability to take up products of abnormal nerve cell metabolism, prevents their accumulation in the tissues and so functions as a "shielding" mechanism, gaining time for the mobilization of "apolar elements" on the one hand, and preventing sudden access of toxic products to the blood stream on the other. This attractively systematized hypothesis is not, however, in keeping with the observations in the mouse, where nerve cell disturbance, as judged by clinical condition, must be profoundly affected.

Elsewhere the author has considered the possibility that rabies virus once it has reached the nervous system may spread interstitially, attacking nerve cells and microglia at the same time from without. This hypothesis based on findings in the rabbit, receives no support from those made in the mouse.

CONCLUSIONS

A comparison of the microglial reaction in rabic encephalitis in the mouse and the rabbit has been made. Whilst it is a prominent feature of the pathological process in the rabbit, beginning when nerve cell damage is possibly absent or only minimal as judged from the Nissl picture, and going on to lamellar cell formation in the advanced stages of the disease, in the mouse there is no generalized microglial reaction at all. Only along the track of the injecting needle is there a reaction and this is identical with that found in control puncture wounds.

Some theoretical considerations of microglial function are discussed.

ACKNOWLEDGMENT

I should like to thank Professor F. Blakemore for facilities offered at the University of Bristol Veterinary Laboratories,Lang ford, and Professor J. M. Yoffey for histological facilities. I am grateful to Miss M. C. Le Bechee for technical assistance and to the Medical Research Council for a grant.

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LEGENDS FOR FIGURES

Fig. 1. (a) Normal cerebellar cortex of rabbit. Microglia is highly branched and shows fine hairlike offshoots. Hortega x 470.

(b) E.502. Shrunken microglia with thickened granular processes, 24 hours after onset of fever. Hortega x 470.

Fig. 2. (a) Microglia of normal cerebral cortex of rabbit. Hortega x 470.

(b) E.424. Twenty-four hours after onset of fever. Microglia shows thickened processes many having globular expansions beset with remains of fine hairlike offshoots. Hortega x 470.

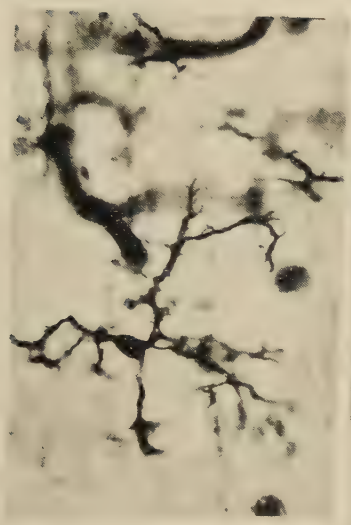
Fig. 3. (a) E.512. Nerve cells in hypoglossal nucleus. These cells are normal or at most show minor thickening of Nissl granules. Giemsa x 350.

(b) E.512. Microglial reaction definite a little distance headward of section (a) Hortega x 500.

Fig. 4. E.459. Rabbit in extremis. Well developed lamellar cells, often applied to capillaries and occasionally (but not especially) to nerve cells. Hortega x 500.

Fig. 5. (a) Severely reacting microglia close to track of inoculating stab in mouse brain. Hortega x 340.

(b) A little further from the stab wound microglia is impregnated but normal in appearance. Further out still it fails to take the silver. Hortega x 340.



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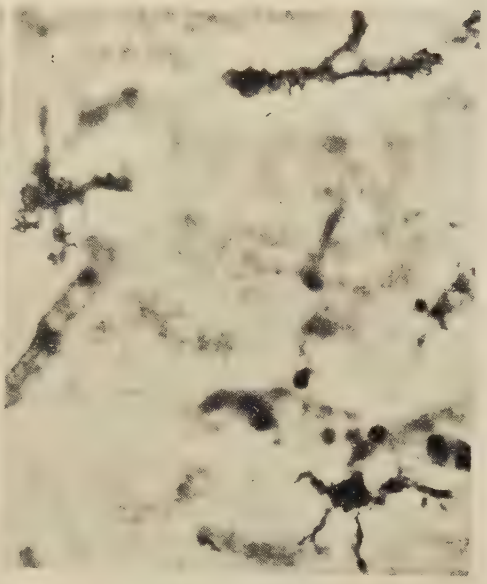

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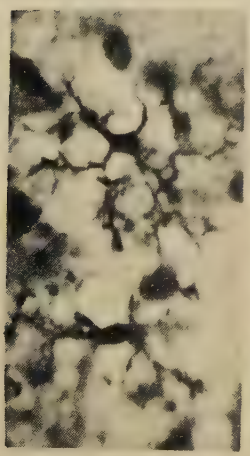

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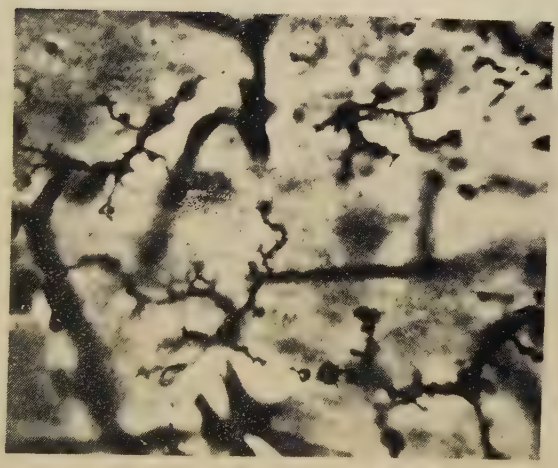

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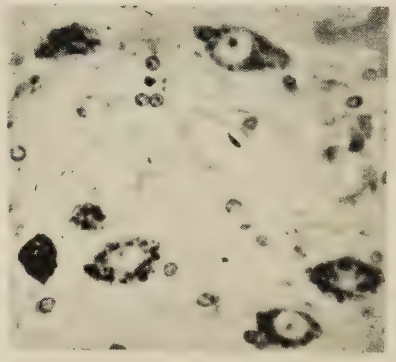

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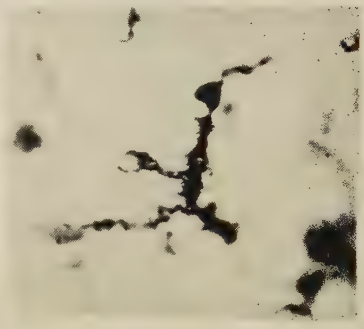

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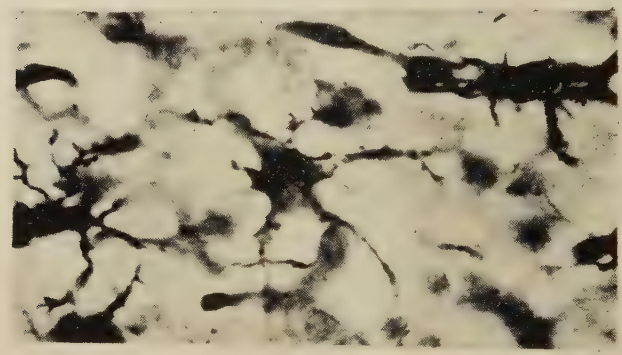

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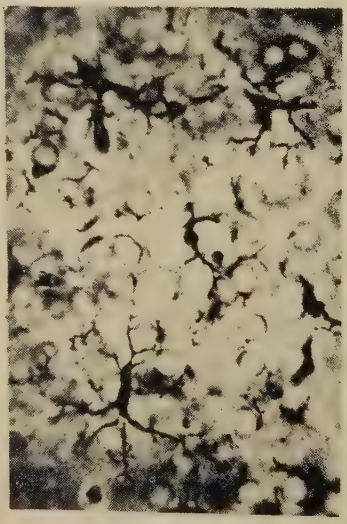

5 (b)

"DEGRADED" STRAINS OF *CLOSTRIDIUM WELCHII* TYPE C ISOLATED FROM SHEEP IN NEW ZEALAND

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INTRODUCTION

Clostridium welchii Type C was first isolated from sheep on the Romney Marsh in England (McEwen, 1930) and was later shown by McEwen and Roberts (1931) and McEwen (1932) to be the cause of an enterotoxaemic condition of sheep. Griver and Bracken (1953) found that the same organism is associated with an acute haemorrhagic enteritis of calves in the United States. Infection with *Cl. welchii* Type C has also been observed in Wales (Montgomerie and Rowlands, 1936), in France (Weinberg, Guillaumie and Staub, 1935) and in Turkey (Weinberg, Guillaumie and Forgeot, 1935) but there are no reports of the isolation of the organism from sheep in New Zealand or Australia.

In 1948, a survey was initiated into hogget mortalities in the Otago and Southland districts in New Zealand. The importance of infections with anaerobic bacteria as a cause of such mortalities in these areas has been appreciated since the early investigations on "braxy-like disease of sheep (particularly hoggets) on turnips", initiated by Gilruth in 1898. Evidence was first obtained in 1931 that sudden deaths amongst hoggets on turnip crops in the South Island of New Zealand could be the result of *Cl. welchii* Type D enterotoxaemia (Gill, 1936). *Cl. chauvoei* infection in hoggets was first recognised in the Otago and Southland districts in 1944 and vaccination against this disease was initiated there in 1947. The term "braxy-like disease" was still commonly applied to all hogget losses in 1948 and this necessitated investigations on individual properties to assess the relative frequency of infection with the various clostridia so that effective control procedures could be applied. Deaths in older sheep from suspected enterotoxaemia or gas gangrene were also investigated.

The purpose of this communication is to record the isolation of "degraded" or variant strains of *Cl. welchii* Type C from the alimentary tract of sheep in New Zealand.

ISOLATION AND CHARACTERS OF THE ORGANISM

Field Reports

In September 1948, a mortality involving fourteen ewes on a property at Kapuka, Southland was investigated by Mr. J. P. Anderson, Veterinarian of the Department of Agriculture. The freshest carcase examined was a full-mouth Romney ewe which was due to lamb in from three to four weeks. The most prominent abnormality was inflammation of the small intestines. Entero-

toxaemia was suspected, and a rib and a sample of the ileal contents were forwarded for laboratory examination.

In May 1949, Mr. C. A. Martin, Livestock Instructor of the Department of Agriculture, investigated a mortality involving four ewes from a flock of one hundred at Tapanui, West Otago and collected specimens from a four-tooth Romney ewe which had been dead for several hours and showed blood-stained froth exuding from the nostrils. The abomasum showed severe inflammation particularly in the region of the pylorus and the first few feet of the small intestine were similarly affected. The tentative diagnosis was enterotoxaemia and a specimen of the ileal contents was submitted for laboratory study.

Bacteriological Studies

For convenience in subsequent descriptions, the first case is referred to as "Robinson" and the second as "Chandler."

The bone marrow from the rib of Robinson's case was sown on the surface of blood agar plates and no bacterial growth was observed after aerobic and anaerobic incubation.

Filtrates of the ileal contents from both cases were tested for lethal toxin by intravenous inoculation into mice. The animals succumbed to doses of 0.4 ml. of filtrate from the Robinson case and 0.2 ml. from the Chandler. The Chandler filtrate was tested for neutralisation by *Clostridium welchii* antitoxic sera Types A, C and D. In each case a mixture containing 4 parts of filtrate and one of antitoxic serum was incubated at 37°C for 30 minutes and inoculated in 0.5 ml. quantities intravenously into mice. The particulars of the sera are as follows:— "A" antitoxic serum No. LX.286: 1 ml. 95 units alpha antitoxin, 165 units theta antitoxin. "C" antitoxic serum No. LX.336: 1 ml. 90 units alpha antitoxin, 190 units theta antitoxin, 850 units beta antitoxin, 2.5 units delta antitoxin. "D" antitoxic serum No. LX.288: 1 ml. 80 units alpha antitoxin. 115 units theta antitoxin, 300 units epsilon antitoxin. In these tests, 0.4 ml. of the filtrate was neutralised by 0.1 ml. of the "C" antitoxic serum but not by 0.1 ml. of either "A" or "D" antiserum.

Turner and Eales (1944) have shown that strains of *Cl. welchii* Type C produce a characteristic haemolytic reaction on ruminant blood agar due to the action of the delta haemolysin and this reaction may serve as a presumptive test for recognising colonies of *Cl. welchii* Type C. They found that the most satisfactory basal medium for the purpose was veal infusion plus Witte peptone agar. Since Witte peptone was not available in New Zealand, tests were made with other peptones using either sheep or ox defibrinated blood. Several classical strains of *Cl. welchii* Type C when tested on veal infusion agar containing one per cent May and Baker peptone plus ten per cent fresh defibrinated ox blood were found to produce the characteristic reaction (Fig. 1) whilst the same basal

medium plus ten per cent sheep blood was unsatisfactory (Fig. 2). Consequently this medium with ox blood added was adopted throughout the survey to facilitate the recognition of colonies of *Cl. welchii* Type C.

The ileal contents of the Robinson and Chandler cases were sown on the surface of veal infusion ox blood agar plates which were incubated anaerobically for 24 hours at 37°C. In both cases, a profuse almost pure growth of colonies morphologically resembling *Cl. welchii* was observed and around these colonies, outside the narrow zone of clear haemolysis was a wide zone of bright pink haemlysis. Six typical colonies from each case were first subcultivated in veal infusion broth and then replated on (a) the standard blood agar, and (b) this medium containing ten per cent "A" antitoxic serum and (c) this medium containing ten per cent "C" antitoxic serum.

The secondary zone of bright pink haemolysis described as characteristic of *Cl welchii* Type C by Turner and Eales (1944) was again produced around all colonies on the standard blood agar plates after 24 hours anaerobic incubation (Figs. 3 and 4). The addition of the "A" antitoxic serum to the medium had a stimulating ing effect on the characteristic secondary zone of haemolysis around the Robinson and Chandler colonies (Fig. 5). The addition of the "C" antitoxic serum to the medium suppressed but did not completely inhibit the haemolytic reaction.

The haemolytic reactions of several strains each of *Cl. welchii* Type A, Type C and Type D, were observed on the same batches of standard blood agar and blood agar plus ten per cent. "A" antitoxic serum. The addition of the "A" antitoxic serum completely suppressed the haemolytic reaction around the colonies of *Cl. welchii* Type A (Figs. 6 and 7) and *Cl. welchii* Type D, whilst the characteristic haemolytic reaction around the colonies of *Cl. welchii* Type C was non-specifically enhanced (Figs. 8 and 9).

Six Robinson strains and six Chandler strains were tested for toxigenicity immediately following isolation and on later occasions. The relative toxicity for mice of filtrates from cultures of these strains in a variety of media following varying periods of incubation was estimated. Filtrates of either six hour cultures in veal infusion peptone glucose (1 per cent) broth plus ten per cent minced sheep heart or twenty-four hour cultures in the same medium plus ten per cent sheep serum compared favourably with others for concentration of lethal toxins and were used in subsequent tests for the identification of the specific toxins elaborated by these strains.

Lecithinase activity of the strains was tested using the techniques of Macfarlane, Oakley and Anderson (1941) and Wagner (1949). The filtrates of the Robinson and Chandler strains were shown to contain alpha toxin (lecithinase) which was neutralised by the *Cl. welchii* Type A antiserum (No. LX.286).

Haemolytic tests were conducted with the filtrates using suspensions of rabbit and sheep red cells. The haemolytic effects of the filtrates on rabbit cells was inhibited by the *Cl. welchii* Type A antiserum. Inhibition of haemolysis of sheep red cells in borate buffer saline pH 8 was effected by Type C antiserum but not by Type A antiserum.

Lethal tests were conducted by the intravenous inoculation of mice with both crude and trypsinized filtrates. Filtrates of six hour cultures in veal infusion peptone glucose broth of the Chandler strains did not kill mice in doses less than 0.1 ml. and in the case of the Robinson strains doses of less than 0.2 ml. were not lethal. The addition of ten per cent serum to the medium did not enhance the toxicity of filtrates of six hour cultures of the Robinson or Chandler strains but did so significantly in both cases when the incubation period was extended to twenty-four hours. Mice inoculated with the larger doses of filtrates usually died within three to fifteen minutes after exhibiting violent spasmodic twitchings immediately prior to death.

The crude and trypsinized filtrates were mixed with each of the three antitoxic sera in the proportion of four parts of filtrate to one of antiserum, the mixtures being then incubated at 37°C for 30 minutes and finally inoculated intravenously into mice in doses of 0.5 ml. The results indicated that 0.4 ml. of the filtrates of the Robinson and Chandler strains was not neutralised by 0.1 ml. of either the Type A antitoxic serum (LX.286) or the Type D antitoxic serum (LX.288) but was neutralised by the Type C antiserum (LX.336).

Crude and trypsinized filtrates of the Robinson and Chandler strains were tested for necrotizing toxins by the intradermal inoculation of 0.2 ml. of filtrates and mixtures of filtrates and antitoxic sera into depilated guinea pigs. The filtrates induced only slight necrotic reactions which were inhibited by each of the three antitoxic sera.

Sheep Experiments

Pathogenicity and toxicity tests conducted in sheep with freshly isolated cultures from the Robinson and Chandler cases have been limited to a few animals. In the first test, a twenty-four hour culture in veal infusion serum broth of a freshly isolated strain from the Robinson case was utilised. One hogget was inoculated intramuscularly in the medial aspect of the thigh with 5 ml. of this culture and a second hogget was similarly inoculated with 5 ml. of culture to which 0.2 ml. of adrenalin HCl solution (1:1000) had been added. A third hogget was inoculated intravenously with 5 ml. of a Seitz filtrate of the same culture. The first hogget showed slight swelling and oedema of the inoculated leg on the following day whilst the hogget inoculated with the culture containing adrenalin showed

severe swelling and oedema extending to below the hock. Neither hogget exhibited obvious systemic disturbance and both recovered from the local reaction within three days. Intravenous inoculation of filtrate caused only minor temporary disturbance in the treated hogget.

In the second test, one hogget received by oesophageal tube, a litre of ewes' milk previously inoculated with 15 ml. of an 18 hour culture of a Robinson strain and another hogget similarly received ewes' milk inoculated with a culture of a highly toxigenic strain of *Cl. welchii* Type C. The ewes' milk had been autoclaved to 15 lb. pressure and then rapidly cooled before being inoculated. The two hoggets remained normal whilst kept under observation for three days following the experimental treatment.

In the third test one hogget was inoculated intravenously with 5 ml. of a filtrate from a 6 hour culture of a Chandler strain. Apart from the development of haemoglobinurea within a few hours of inoculation, the animal exhibited no serious disturbance.

In the fourth test, the bacteria from cultures of a Robinson strain, a Chandler strain, and a toxigenic strain of *Cl. welchii* Type D were suspended in cows' milk and administered by oesophageal tube to three lambs aged between nine and twelve weeks. The centrifuged organisms from 400 ml. quantities of 16-hour broth cultures were resuspended in 400 ml. of autoclaved milk. The lambs had been separated from the ewes for 24 hours prior to dosing and were returned to them immediately afterwards. The lambs were not affected by the experimental treatment.

Investigations at the Wellcome Research Laboratories, England

In order to obtain precise information on the identity of the lethal toxins elaborated by the Robinson and Chandler strains it was necessary to prepare concentrated dried toxins and a greater variety of antitoxic sera than was available to us (Oakley, 1943). The application of immunological procedures has been recommended (Dalling and Ross, 1938), for the demonstration of traces of toxin which cannot be detected by direct methods. We are greatly indebted to Dr. R. F. Montgomerie for arranging for more extensive studies to be undertaken on these strains at the Wellcome Research Laboratories, England, and for his permission to present here the following summary of the results obtained.

Five hour culture filtrates of the Robinson and Chandler strains were shown to contain traces of alpha toxin and relatively large amounts of delta haemolysin (1 to 2 delta units per ml. of filtrate.). The presence of other toxins could not be established by direct methods so two horses were used to determine whether filtrates of the Chandler strain contained other antigens. The sera of these horses were shown to contain no beta antitoxin prior to immunisation. Following hyperimmunisation with filtrates of the cultures of the Chandler strain, each produced a small quantity of beta

antititoxin, one reaching a titre of 2 units per ml. and the other 11 units per ml. Antitoxins were also developed against the other antigens present in the culture filtrate, a good response being observed to delta toxin, with reasonably good responses to alpha and kappa. No lambda antitoxin was produced.

The immunological test thus indicated that the Chandler strain did produce a trace of beta toxin in culture filtrates in addition to the production of alpha, delta and kappa, but no lambda and so could be classified as a "degraded" strain of *Cl. welchii* Type C.

DISCUSSION

The haemolytic reaction around colonies of the Robinson and Chandler strains on ox blood agar had first suggested that these organisms might be *Cl. welchii* Type C. Whilst strains of *Cl. welchii* Type B may produce small but demonstrable amounts of delta haemolysin, Turner and Eales (1944) have not observed this characteristic reaction with Type B strains. Further evidence that the Robinson and Chandler strains were capable of elaborating delta toxin was afforded by the effects of Type A and Type C antisera when added to ox blood agar plates and by haemolytic tests with sheep red cells. Tests with egg-yolk emulsion and haemolytic tests with rabbit red cells indicated that alpha toxin (lecithinase) was also produced by these strains. Skin tests did no more than reveal the presence of minute quantities of toxins, probably of the alpha variety, although *Cl. welchii* Type C strains may also elaborate beta and kappa necrotizing toxins (Oakley and Warrack, 1953).

Lethal tests with mice demonstrated that the strains were of low toxigenicity, filtrates of cultures incubated for short periods being more potent than those of longer incubation unless serum had been added to the medium. The lethal activity of filtrates of *Cl. welchii* Type C strains may be due to alpha, beta, gamma, delta, theta (?) and kappa toxins (Oakley and Warrack, 1953). Dalling and Ross (1938) had first demonstrated that beta and delta toxins are present in culture filtrate after only a few hours' incubation, but whilst the delta toxin apparently disappears rapidly with further incubation, the beta toxin is relatively stable during prolonged incubation. Turner and Eales (1944) have observed that the addition of serum enhances the production of delta toxin in most media examined and may prevent rapid deterioration of the elaborated delta toxin. Whilst the presence of beta toxin in culture filtrate of the Robinson and Chandler strains was not confirmed by the intradermal tests in guinea-pigs, mice inoculated with filtrates showed spasmodic convulsions not dissimilar to those reported as characteristic of the beta toxin by Dalling and Ross, (1938).

These strains when examined immediately following isolation failed to produce beta toxin in amounts that could be detected by direct methods, even when cultivated under conditions considered

as optimal for the production of this specific toxin. However, the immunological test with the Chandler strain indicated that a trace of beta toxin was present in culture filtrates, suggesting that these strains were *Cl. welchii* Type C with an inherently poor capacity to elaborate beta toxin.

There have been several reports of strains of *Cl. welchii* Type C losing their capacity to produce particular toxins following repeated cultivation on artificial media. Dalling and Ross (1938) reported that a Type B strain had lost the capacity to elaborate the epsilon toxin and further that a Type C strain on one occasion produced alpha and delta toxins but no beta toxin. Taylor (1940) also described the loss of capacity to produce beta toxin by certain cultures of a Type C strain. Turner and Eales (1944) had observed this phenomenon with the same strain and reported the isolation from it of a variant which was able to produce the characteristic haemolytic reaction on blood agar but was unable to produce beta toxin.

The evidence is not conclusive that these strains of *Cl. welchii* Type C were responsible for the deaths of the ewes from whose ileal contents they were recovered. Whilst blood agar plates inoculated with ileal contents from the Robinson and Chandler strains exhibited a profuse, almost pure growth following incubation under anaerobic conditions, proliferation of these bacteria in the contents of the small intestine may have occurred a short time prior to or following the death of these ewes.

Cl. welchii Type C had not been recognised previously in New Zealand and the isolation of these "degraded" strains posed questions as to their possible rôle in sheep mortalities and to the possible existence of more toxigenic variants of Type C in this country. During the course of the investigation of clostridial infections of hoggets and older sheep in Otago and Southland over the period 1948 to 1951, additional strains of *C. welchii* of similar characters were not isolated. Consequently further opportunities to assess the aetiological significance of these "degraded" or variant strains of *Cl. welchii* Type C in ovine mortalities in New Zealand have not been available.

CONCLUSIONS

Cl. welchii Type C had not been recognised in New Zealand prior to 1948.

During the course of investigations into clostridial infections of sheep in the Otago and Southland districts over the period 1948 to 1951, "degraded" strains of *Cl. welchii* Type C were isolated from the ileal contents of two ewes that had died from natural disease.

The characteristic haemolytic reaction on ox blood agar produced by the delta toxin facilitated the recognition and isolation of these "degraded" strains of *Cl. welchii* Type C.

These strains were shown to be of low toxigenicity and pathogenicity for experimental sheep.

The Robinson and Chandler strains were shown by direct methods to be capable of elaborating alpha and delta toxins and the presence of beta and kappa toxins in culture filtrates of the Chandler strain was established by immunological procedures.

The aetiological significance of these "degraded" or variant strains of *Cl. welchii* Type C in ovine mortalities remains to be clarified.

ACKNOWLEDGMENT

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LEGENDS FOR FIGURES

Fig. 1

Cl. welchii Type C colony on ox blood agar showing characteristic haemolytic reaction.

Fig. 2

Cl. welchii Type C colony on sheep blood agar. No secondary zone of partial haemolysis apparent.

Fig. 3

Robinson strain on ox blood agar showing haemolytic reaction.

Fig. 4

Chandler strain on ox blood agar showing haemolytic reaction.

Fig. 5

Chandler strain on ox blood agar plus 10 per cent Type A antitoxic serum, showing non specific enhancement of haemolytic reaction.

Fig. 6

Cl. welchii Type A colonies on ox blood agar.

Fig. 7

Cl. welchii Type A colonies on ox blood agar plus 10 per cent Type A antitoxin serum, showing inhibition of haemolysis.

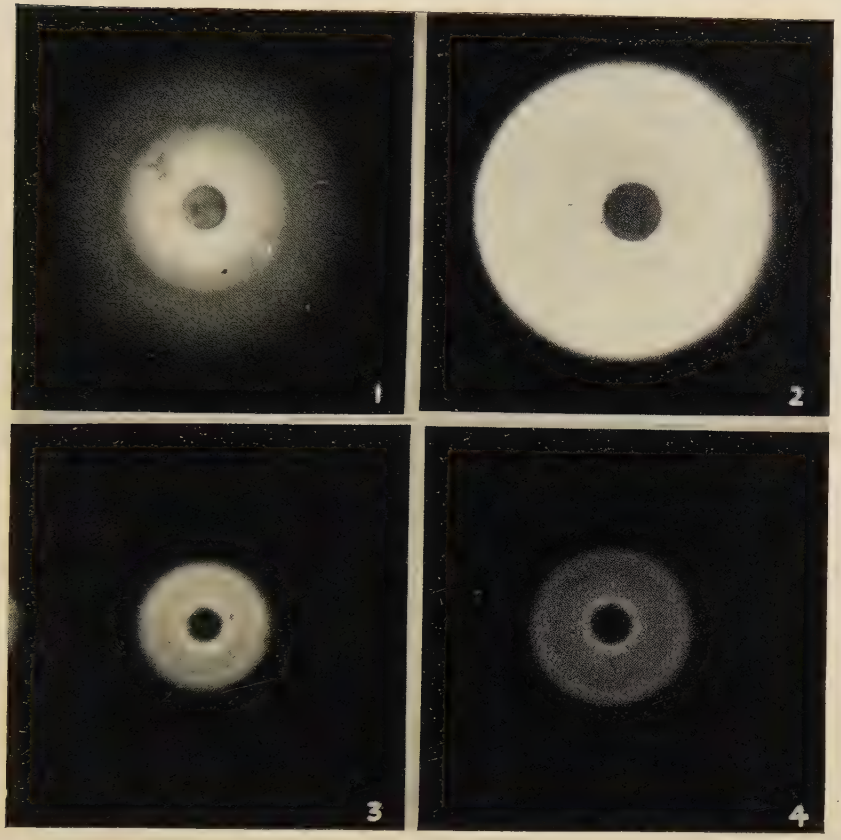
Fig. 8

Cl. welchii Type C colony on ox blood agar showing haemolytic reaction.

Fig. 9

Cl. welchii Type C colony on ox blood agar plus 10 per cent Type A antitoxic serum showing non specific enhancement of haemolytic reaction.

CLOSTRIDIUM WELCHII STRAINS FROM SHEEP



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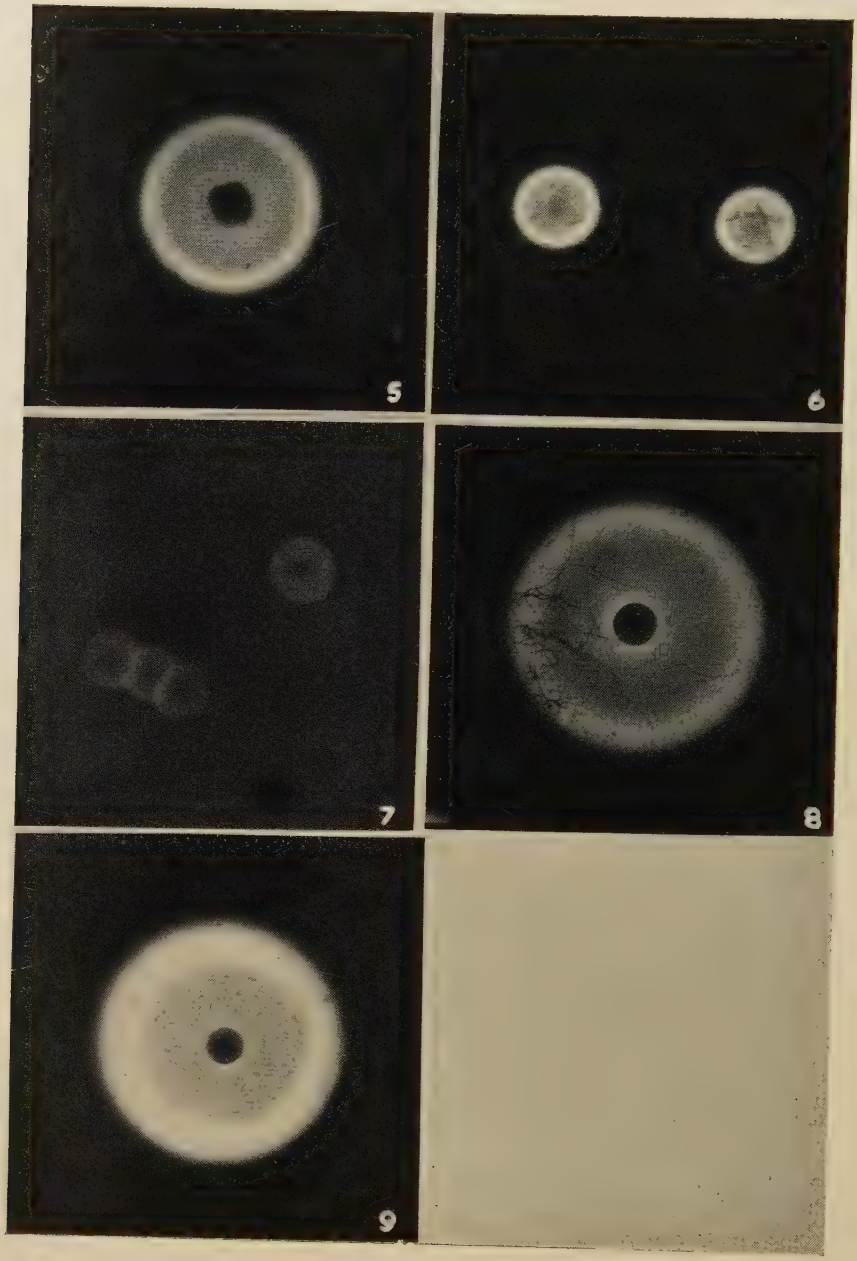
CLOSTRIDIUM WELCHII STRAINS FROM SHEEP



SERUM LEVELS OF PENICILLIN, DIHYDROSTREPTOMYCIN, CHLORAMPHENICOL, AUREOMYCIN AND TERRAMYCIN IN CHICKENS

By

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INTRODUCTION

During some studies on the treatment of *Salmonella gallinarum* infection, information was required as to the serum levels obtainable in chickens following the administration of chloramphenicol. A search through the literature revealed a paucity of published information on serum levels in chickens of penicillin, dihydrostreptomycin, aureomycin and terramycin as well as of chloramphenicol. It was decided, therefore, to carry out such observations, the results of which are reported in this paper. Observations were also made on the elimination of these chemotherapeutic agents in chicken faeces and on the serum levels that were obtained in chickens that had been fed or starved and then given terramycin or chloramphenicol by mouth.

MATERIALS AND METHODS

Chickens

The chickens used were Brown Leghorn x Barred Rock pullets weighing approximately 2¼ kg.

Chemotherapeutic Agents

Penicillin. Crystalline sodium penicillin G of a potency of 1617 units/mg. was used.

Procaine penicillin. Procaine penicillin was used in two forms. In one, 'Distaquaine', the suspending agent was a mixture of sodium carboxymethylcellulose and a sorbitan mono-oleate polyoxyalkylene derivative; the other was suspended in arachis oil. Both forms were stated to contain 300,000 units per ml. Since all serum levels are recorded in this work as microgrammes/ml. (μg./ml.), 1617 units were considered as being equivalent to 1 mg. of penicillin in both these preparations.

Dihydrostreptomycin. Dihydrostreptomycin sulphate was used, the results being expressed in terms of dihydrostreptomycin base.

Chloramphenicol. For oral administration an aqueous suspension of chloramphenicol was employed. A soluble form (Allen and Hanbury) was used for intramuscular injection.

Aureomycin. Aureomycin hydrochloride in aqueous solution or suspension was employed for oral administration. A solution in distilled water was used for intravenous and intramuscular injection. Since intramuscular administration is often accompanied by pain in other animals, a 1 per cent solution of procaine was first injected into the area chosen for intramuscular administration. Chickens so injected showed no evidence of pain and no swelling was noted at the site of injection.

Terramycin. Tablets containing terramycin hydrochloride were employed for oral administration and an aqueous solution for intravenous and intramuscular injection.

Serum Samples

Blood in 2 ml. amounts was removed from the wing vein of chickens by means of a sterile syringe at different intervals of time after administration of a chemotherapeutic agent. The samples were allowed to clot, stored at 4°C. overnight, after which the serum was removed for assay.

Assay Procedure

A modification of the original plate technique of Florey *et. al.* was used for all assays.

Preparation of plate. A large plate was made with two sheets of glass, a rectangular frame of wood, 32 x 25 cm., forming the sides. The frame was first sealed to the glass sheet forming the bottom of the plate with a small amount of nutrient agar. A basic layer consisting of 500 ml. of nutrient agar was then poured into the plate and, as soon as it had set, a second layer, the flood layer, consisting of 250 ml. of agar was poured on to it. The agar comprising the flood layer had been held previously at 50°C. and a suitable amount of a bacterial culture, very sensitive to the agent that was being assayed, added to it immediately before pouring. When the agar had set firmly, a cork-borer (No. 4) was used to punch out holes at suitable distances apart. The bottom of the holes were sealed with a small amount of nutrient agar and completely filled with different samples of sera to be assayed. Each hole held approximately 0.2 ml. A series of holes was also filled with suitable two-fold falling dilutions in chicken serum of the chemotherapeutic agent under study. The plate was incubated at 30°C. for 18 hours and the diameters of the zones of inhibition measured with calipers. The actual concentration of chemotherapeutic agent in each sample was then determined by reference to a graph prepared by plotting the log. of the known concentrations of the chemotherapeutic agent against the diameter of the zones of inhibition they produced.

Medium. The formula for the nutrient agar was: Peptone 5.0 g.; Lemco 3.0 g.; Agar (Davis, New Zealand) 15.0 g.; Water 1000 ml.

The pH. of the medium was adjusted to 5.6 for aureomycin and terramycin assay, 7.2 for penicillin and chloramphenicol assay and 8.2 for dihydrostreptomycin assay. The concentration of agar in the flood layer of medium for dihydrostreptomycin assay was reduced to 1.0 per cent.

Sensitive bacteria for assay plate. For chloramphenicol assay an 18 hour broth culture of *Bacterium friedlanderi* (Klebsiella 41) in a concentration of 1:500 in the flood agar was used. In the case of the other agents a spore suspension of *Bacterium subtilis* was employed in a concentration of approximately 10^6 viable spores per ml. of flood agar.

By the method described above it was possible to detect concentrations in serum of as low as 0.05 μg./ml. of penicillin, 0.1 μg./ml. of aureomycin, 0.2 μg./ml. of terramycin and 1.0 μg./ml. of dihydrostreptomycin and chloramphenicol.

The Estimation of Chemotherapeutic Agents in Food and Faeces

Since chicken faeces when voided are usually contaminated with urinary material it was not possible to estimate the total amount of chemotherapeutic agent eliminated through the alimentary tract following oral administration. Instead the faecal concentration was measured in the following way and compared with the concentration given in the food.

Two chickens were starved for 18 hours and then fed 150 g. of dry mash into which was mixed 100 mg. of chemotherapeutic agent dissolved in 220 ml. of water. The chickens were killed 4 hours later and approximately 1. 0g. of the faeces immediately in front of the urogenital sinus removed, agitated in 10 ml. of sterile water and centrifuged at 3,500 r.p.m. for 30 minutes. The concentration of the chemotherapeutic agent in the suspended fluid and in ten-fold dilutions of it was then assayed by the plate method. A similar procedure was carried out with the food.

It was known from previous studies that food would have reached the rectum by the time examination was made and this was confirmed by the recovery of some of the drugs in full concentration.

RESULTS

Serum levels after Oral or Intramuscular Administration

Unless otherwise stated, the results quoted below for each dose level of each chemotherapeutic agent are the average four chickens. *Penicillin.* The results of administering penicillin intramuscularly and orally and procaine penicillin intramuscularly at dose levels of 50, 25, 12 and 6 mg/kg. are shown in Table I. Since little difference was noted between the results for procaine penicillin in arachis oil and for the 'Distaquaine' preparation they are not considered separately; the figures quoted are those for 'Distaquaine.'

TABLE I

SERUM CONCENTRATIONS (μG/ML) FOLLOWING ADMINISTRATION OF PENICILLIN

Dose (mg./kg.)	*Kind of penicillin and route*	*Hours after Administration*								
		½	1	2	4	6	8	11	14	24
50	Procaine, intramuscular	3.7	3.5	3.7	2.4	1.9	1.8	1.0	0.6	0.2
	Aqueous, intramuscular	36.0	22.0	7.2	1.0	0.2	0.06	0	0	0
	Aqueous, oral	—	1.0	0.4	0.05	0	0	0	0	0
25	Procaine, intramuscular	3.2	2.8	3.2	2.0	1.0	0.7	0.3	0.2	0.05
	Aqueous, intramuscular	22.0	10.0	5.0	0.4	0.02	0	0	0	0
	Aqueous, oral	—	0.3	0.2	0	0	0	0	0	0
12	Procaine, intramuscular	1.6	1.8	2.2	1.2	0.7	0.2	0.1	0.1	0.05
	Aqueous, intramuscular	9.0	4.0	1.0	0.1	0	0	0	0	0
	Aqueous, oral	—	0.15	0.1	0	0	0	0	0	0
6	Procaine, intramuscular	1.0	0.9	0.8	0.6	0.3	0.1	0.05	0	0
	Aqueous, intramuscular	6.0	1.3	0.25	0.05	0	0	0	0	0
	Aqueous, oral	—	0.1	0.05	0	0	0	0	0	0

The figures for the serum concentrations represent the mean of four chickens.

Procaine penicillin was absorbed slowly and was detectable in the serum of all the chickens at 24 hours except those injected with 6 mg/kg. Penicillin in aqueous solution was absorbed much more rapidly after intramuscular injection and high serum concentrations were found at intervals up to 2 hours. The amount of penicillin in the serum then decreased rapidly and at the lower dose levels was no longer detectable at 4 hours after injection. Only a very small amount of penicillin was absorbed after oral administration and that was excreted rapidly.

Dihydrostreptomycin. The average serum levels observed following the intramuscular injection of dihydrostreptomycin sulphate in amounts of 50, 25, 12 and 6 mg/kg. are shown in Table 2.

TABLE 2

SERUM CONCENTRATIONS (μG/ML) FOLLOWING INTRAMUSCULAR ADMINISTRATION OF DIHYDROSTREPTOMYCIN

Dose (*mg./kg.*)	*Hours after administration*							
	1	2	4	6	8	11	14	24
50	64.0	32.0	22.4	12.0	5.8	2.4	1.0	0
25	28.5	21.0	13.0	5.8	3.8	1.8	0.8	0
12	21.0	12.0	6.2	3.7	2.0	0	0	0
6	12.0	5.6	3.9	1.8	1.0	0	0	0

The agent was absorbed rapidly. High serum concentrations, 64 μg/ml. in chickens that received 50 μg/kg., were observed within 1 hour. Excretion took place rather slowly and concentrations were detected at 14 but not 24 hours in the sera of the chickens that received 25 and 50 mg/kg. It was not detected after 8 hours in the sera of chickens that had received 12 and 6 mg/kg.

No dihydrostreptomycin was found in the sera of four chickens that had been given 50 mg/kg. of dihyrostreptomycin sulphate by the oral route.

Chloramphenicol. The average serum levels of chloramphenicol in chickens after intramuscular and oral administration is shown in Table 3.

TABLE 3

SERUM CONCENTRATIONS (μG/ML) FOLLOWING ADMINISTRATION OF CHLORAMPHENICOL

Dose (*mg./kg.*) *and route*		*Hours after administration*							
		1	2	4	6	8	11	14	18
200	oral	14.5	16.0	17.0	18.0	14.0	12.2	8.0	0
100	oral	16.5	16.0	16.0	10.2	4.7	0	0	0
50	intramuscular	32.0	30.5	25.0	9.8	2.0	0	0	0
	oral	12.0	11.0	2.0	0	0	0	0	0
25	intramuscular	13.6	9.2	2.6	0	0	0	0	0
	oral	7.4	6.2	1.0	0	0	0	0	0
12	intramuscular	5.2	2.6	0	0	0	0	0	0
	oral	3.5	3.3	0	0	0	0	0	0
6	intramuscular	2.6	1.5	0	0	0	0	0	0

Following intramuscular injection chloramphenicol was rapidly absorbed into the blood stream and rapidly disappeared therefrom. It was no longer detected 2 hours after administration in the serum of chickens that received 12 or 6 mg/kg., after 4 hours in those that received 25 mg/kg. or after 8 hours in those that received 50 mg/kg.

Chloramphenicol was fairly well absorbed after oral administration, the highest serum concentrations obtained at a dose level of 50 mg/kg. being approximately 12 μg/ml. Very little increase in blood concentrations was noted when the dose level was increased to 200 mg/kg. but serum concentrations were maintained for a much longer time, the agent being still detectable in the serum at 16 hours compared with 4 hours after a dose of 50 mg/kg. A considerable variation was noted between the serum concentrations obtained in different chickens which were given the same dose of chloramphenicol orally. The variation was much more pronounced with chloramphenicol than with any of the other agents that were administered by the oral route.

Aureomycin. The serum levels observed after administering aureomycin at dose levels of 200, 100, 50, 25, 12 and 6 mg/kg. orally, 50, 25, 12 and 6 mg/kg. intramuscularly and 25 mg/kg. intravenously are shown in Table 4.

TABLE 4

SERUM CONCENTRATIONS (μG/ML) FOLLOWING ADMINISTRATION OF AUREOMYCIN

Dose (mg./kg.) and route		*Hours after administration*							
		1	2	4	6	8	11	14	24
200	oral	1.9	3.2	2.0	0.9	0.5	0.25	0.2	0.07
100	oral	0.8	2.0	1.3	0.5	0.3	0.2	0.2	0.08
50	oral	0.4	0.6	0.7	0.4	0.2	0.1	0.05	0.04
	intramuscular	1.2	0.9	0.6	0.5	0.4	0.3	0.2	0.1
25	oral	0.18	0.2	0.2	0.2	0.1	0.04	0.04	0.04
	intramuscular	0.6	0.5	0.3	0.3	0.2	0.1	0.08	0.04
	intravenous	26.0	20.0	15.0	6.4	4.5	2.4	2.4	0.05
12	oral	0.14	0.2	0.14	0.17	0.1	0.06	0.04	0
	intramuscular	0.5	0.4	0.25	0.2	0.14	0.07	0.06	0.03
6	oral	0.1	0.1	0.07	0.07	0.07	0.04	0	0
	intramuscular	0.25	0.2	0.08	0.08	0.07	0.07	0.06	0

Aureomycin was absorbed very poorly after intramuscular and oral administration. For example, at the 25 mg/kg. dose level, the highest serum concentration obtained was 0.6 μg/ml. after intramuscular and 0.2 μg/ml. after oral administration; the highest serum concentration after intravenous injection at this dose level was 25 μg/ml. Although aureomycin was poorly absorbed it was

excreted very slowly and detectable amounts were usually present in the serum 24 hours after administration.

Terramycin. The serum concentrations of terramycin that were found in chickens following the oral administration of 200, 100, 50 and 25 mg/kg., the intramuscular administration of 50, 25, 12 and 6 mg/kg. and the intravenous administration of 25 mg/kg. are illustrated in Table 5.

TABLE 5
SERUM CONCENTRATIONS (μG/ML) FOLLOWING ADMINISTRATION OF TERRAMYCIN

Dose (mg./kg.) and route		*Hours after administration*							
		1	2	4	6	8	11	14	24
200	oral	1.1	2.0	2.0	1.8	2.5	2.4	2.4	0.6
100	oral	1.4	2.0	1.4	1.4	1.8	1.4	1.0	0.4
50	oral	0.5	0.7	0.6	0.4	0.2	0.2	0.2	0.1
	intramuscular	15.0	17.5	17.5	17.5	17.0	17.5	17.5	5.0
25	oral	0.2	0.2	0.2	0.4	0.2	0.1	0.1	0.1
	intramuscular	10.0	12.0	10.0	10.0	9.2	8.0	6.3	1.5
	intravenous	27.0	17.0	12.0	5.6	4.5	2.3	1.3	0
12	intramuscular	3.3	4.6	4.6	5.4	5.0	4.5	3.2	1.2
6	intramuscular	2.3	2.0	1.4	1.6	1.2	0.5	0.4	0.2

Terramycin was absorbed very poorly from the alimentary tract, the highest serum concentration observed at the 25 mg/kg. level was 0.4 μg/ml. In contrast it was well absorbed after intramuscular injection, concentrations as high as 12 μg/ml. being noted at the 25 mg/kg. dose level. It was very slowly excreted and was usually demonstrable 24 hours after administration at the dose levels studied.

The Concentration of Chemotherapeutic Agents in the Faeces after Oral Administration

The average concentrations of penicillin, dihydrostreptomycin, chloramphenicol, aureomycin and terramycin found in the food, rectal contents and serum of chickens 4 hours after commencing feeding on a diet comprising 100 mg. of chemotherapeutic agent dissolved in 220 ml. of water to every 180 g. of mash is shown in Table 6.

Similar concentrations of dihydrostreptomycin and terramycin, approximately 200 μg/ml., were found in both faeces and food; no streptomycin and only 1.0 μg/ml. of terramycin were found in the serum. A somewhat lower concentration of penicillin and aureomycin was found in faeces than in food, 80 μg/ml. compared with

TABLE 6
THE CONCENTRATION OF CHEMOTHERAPEUTIC AGENTS IN THE FAECES FOLLOWING ORAL ADMINISTRATION

Agent	*Concentration (μg./ml.) in*		
	Food	*Faeces*	*Blood*
Penicillin	200	80	0.05
Dihydrostreptomycin	240	320	0
Chloramphenicol	210	0	11.0
Terramycin	210	200	1.0
Aureomycin	180	80	1.0

The figures for the concentration in the faeces and serum are the mean of two chickens.

approximately 200 μg./ml. It would be difficult to say whether this indicated that some destruction of these two agents had occurred or whether the results were an expression of the inaccuracies inherent in this type of experiment. Only 0.05 μg/ml. of penicillin and 1.0 μg/ml. of aureomycin were found in the serum. Quite different results were observed with chloramphenicol. The food contained approximately 200 μg/ml. of chloramphenicol but none was detected in the faeces; 11.0 μg/ml. was found in the serum.

The Effect of Feeding and Starving on the Absorption of Terramycin and Chloramphenicol from the Alimentary Tract

The average concentration of terramycin observed in the serum of 8 chickens that had been starved for 18 hours, before being given that agent orally in a dose of 100 mg/kg. and in the serum of 8 chickens that had been starved for 18 hours, fed for 1 hour to ensure that their crops were very full of food, and then given a similar dose of terramycin is illustrated in Fig. 1. The serum concentrations of terramycin in the chickens that were fed were almost twice as high as in those that had had no food. Quite different results were obtained in a similar experiment with chloramphenicol (Fig. 2) ; the average serum concentrations of the starved chickens were almost twice as high as those of the fed birds.

Because they had previously been starved the crops of the fed chickens contained much more food than did those of most of the chickens used in the other experiments described in this paper.

DISCUSSION

The results of administering penicillin, streptomycin, chloramphenicol, aureomycin and terramycin to chickens by the oral, intramuscular and intravenous routes, described above and summarized in Fig. 3, resemble, in the main, those that have been found in man and other animals and require little discussion.

A comparison between the results observed following the intramuscular injection of aureomycin and terramycin is of interest;

although prolonged serum levels were obtained with both agents, the serum levels of terramycin were very much higher than those of aureomycin. This poor absorption of aureomycin could not be attributed to the procaine that preceded the intramuscular injection of aureomycin since similar results were obtained when procaine was not used. Irregular results have been noted by others following the intramuscular administration of aureomycin to other animals and have been attributed to the local effects of the aureomycin. It is possible that a similar explanation may account for the results in chickens although no obvious local reaction was noted following intramuscular injection.

The serum concentrations observed in chickens following the oral administration of penicillin, aureomycin and terramycin were much lower than those that have been reported in man and other animals. The low concentrations were not due to the destruction of these agents in the gut but were probably due to poor absorption since they were found in very high concentrations in the faeces, a fact which may be related to the very short length of the alimentary tract of chickens compared with most mammals. The feeding and starving experiments supported this view since better absorption occurred when the alimentary tract was well filled with food than when it was empty; the presence of food would prolong the time spent by the terramycin in the alimentary tract and permit a greater opportunity for absorption. The quite different results obtained with chloramphenicol may be explained as follows. Some destruction of this agent is known to take place in the alimentary tract of mammals. Since none was found in the faeces of chickens after oral administration it is probable that the lower serum concentrations found in the fed than in the starved chickens may be because greater opportunity occurs for the destruction of chloramphenicol in the full than in the empty alimentary tract.

The fact that aureomycin and terramycin are very poorly absorbed following oral administration and that chloramphenicol, although fairly well absorbed, is rapidly excreted is a formidable obstacle to the use of these agents in the treatment of disease in chickens since it is desirable from an economic point of view when large numbers of chickens have to be treated, and particularly when the chemotherapeutic agent is only bacteriostatic, to be able to administer the agent mixed in the food or water. It is probable that terramycin intramuscularly would be the most useful of these three agents for the treatment of valuable chickens or turkeys.

SUMMARY

The persistence and levels of penicillin, procaine penicillin, dihydrostreptomycin, chloramphenicol, aureomycin and terramycin in the serum of chickens after oral, intramuscular and intravenous administration has been investigated.

The results for penicillin, procaine penicillin and dihydrostreptomycin given by intramuscular injection resembled those found in man and other animals. No streptomycin and only a small amount of penicillin was absorbed after oral administration.

Aureomycin and terramycin were absorbed very poorly after oral administration but usually persisted for 24 hours. Low serum levels of aureomycin were also found after intramuscular injection; much higher ones were noted in the case of terramycin.

Chloramphenicol was absorbed very much better from the alimentary tract than were the other agents. It persisted in the serum, however, only for a relatively short period after oral or intramuscular administration.

After oral administration all or nearly all of the dihydrostreptomycin, penicillin, aureomycin and terramycin but none of the chloramphenicol was excreted in the faeces.

More terramycin, but less chloramphenicol, was absorbed from a full than from an empty alimentary tract.

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THE ATTENUATION OF *MYCOBACTERIUM TUBERCULOSIS VAR. MURIS* (THE VOLE ACID-FAST BACILLUS)

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INTRODUCTION

The discovery by Wells (1937) of an acid-fast bacillus in voles prompted a number of investigations into the pathogenicity of the organism and its possible use as an immunising agent. Griffith (1942), Corper and Cohn (1943) and Murochasi, Seki, Takano and Yoshida (1952) in experiments with guinea-pigs showed that the organism appeared to be more pathogenic than B.C.G. Griffith (1942), Wells (1946) and Young and Paterson (1947) found that the organism was pathogenic for rabbits when given in large doses intravenously, producing lesions resembling those caused by the bovine type of tubercle bacillus. Wells (1946) reported that in human subjects, following multiple puncture inoculation of a suspension of the organism, some considerable local lesions developed in certain patients but he considered the dosage used greater than would be required for immunising purposes.

Immunity tests on guinea-pigs conducted by Corper and Cohn (1943) led those authors to the conclusion that the organism had a dangerous tendency to spread from the site of inoculation and that it was not superior in immunising properties to avirulent strains of mammalian tubercle bacilli. Wahlgren, Olin and Widstrom (1944) found that the vole bacillus was at least equal to B.C.G. as an immunising agent for guinea-pigs and suggested that a longer interval between the giving of the challenge dose and slaughter might have shown a greater difference between the protective power of B.C.G. and that of the vole bacillus in favour of the latter. Young and Paterson (1949) found that selected strains of vole bacillus were of value in immunising cattle against tuberculosis and that such strains did not produce progressive infection in the vaccinated animals. Hall and Wylie (1952) compared the vole bacillus and B.C.G. by multiple puncture administration to human subjects and concluded that the vole bacillus produced a slightly greater and more persistent reaction than did B.C.G.

The results of these investigations into the pathogenicity and immunising properties of the organism suggested the possibility of attenuating it to render its use as a vaccine more safe. The early work of Calmette and Guérin (1908) and that of Calmette, Guérin and Weil-Hallè (1924) with B.C.G. suggested that rapid subcultivation of the vole bacillus on bile salt medium might achieve this end. Furthermore, Urquijo (1941) showed that while prolonged

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subcultivation on glycerol potato medium failed to attenuate an avian strain of tubercle bacillus, the same strain subcultivated on glycerol bile potato medium was rendered completely avirulent in five years.

MATERIAL AND METHODS

All the experiments were conducted with a recently isolated strain of vole bacillus, No. LV 2401, obtained from Dr. A. Q. Wells. In the attenuation experiments, the organism was serially subcultivated in parallel on two media, viz. (a) Lowenstein-Jensen medium without glycerol and (b) the same medium with the addition of 0.25 per cent sodium taurocholate. Subcultures were made 50 times at intervals varying from 7 to 21 days over a total period of 671 days.

Pathogenicity tests were made by inoculating rabbits intravenously with suspensions prepared from culture not more than 14 days old. It was found in the course of the experiments that 1.25mg. was a satisfactory dose to use in order to reveal relatively small variations in virulence. The rabbits used weighed between 1,250 g. and 1,500 g. and animals of comparable weights and ages were used for each set of inoculations. The pathogenicity tests were made in parallel, one rabbit or a pair of rabbits being inoculated with a particular generation grown on plain medium and another rabbit or pair of rabbits being given the same dose of the same generation grown on bile salt medium. Rabbits were killed two months after inoculation, except that when a pair of animals was inoculated with a particular strain, one of the pair was allowed to live to determine whether its subsequent behaviour would afford any additional indication of alteration in virulence of the strains. At post mortem examination, all gross lesions were noted and representative portions of the lungs were subjected to histological examination.

RESULTS

Cultural Observations

Vole bacillus strain LV 2401 conformed generally with the characters of the murine type of tubercle bacillus as defined by Griffith (1942) and Wells (1946) in so far as its morphological and cultural appearances were concerned. However, very much more rapid growth was obtained than that reported by Griffith or Wells, colonies being clearly visible in the early subcultures within four or five days after inoculation. At the thirtieth generation, a good growth was present five days after inoculation of the tubes. In all cases typical vole bacilli were seen in Ziehl-Neelsen stained culture films, although as noted by Birkhaug (1946), "shepherd's crook" forms were not numerous.

The growth on plain medium throughout all subcultivations was dry, sharply defined from the underlying medium and raised

into heaps. Colonies were large, rough, with stippled surfaces and in many cultures appeared like coils of worm casts piled up. Chromogenicity was marked, the colonies being a dark yellow, almost orange hue. These colonies were easily detached from the surface of the medium with a platinum loop but were difficult to emulsify in saline solution. Their edges were sharply defined.

The growth on bile salt medium was moist, spreading and parchment-like, tending to cover the entire surface of the slope in contrast to the heaped up growth on plain medium. The colonies were small, hemispherical and shiny. While not typically "smooth", they did not present a "rough" appearance, but were finely stippled. The individual colonies were surrounded by a veil-like periphery which extended into the surrounding medium with no clear line of demarcation. These colonies were only detached from the surface of the medium with difficulty but were readily emulsified in saline solution. Young cultures were devoid of pigment but as they aged a yellowish tinge became evident. After about six months growth there was a slight tendency to heaping of colonies in these cultures but not to the extent observed in plain medium cultures. There was no evidence of dissociation in either the bile salt or plain medium cultures.

Virulence Tests

The results of these tests may be described conveniently by dividing the animals into two groups, viz., Group I comprising

TABLE 1

DETAILS OF VIRULENCE TESTS ON RABBITS

GROUP I

Generation	*Period of growth (days)*	*Dose (mg.)*	*Fate of test animals (days)*
20B	353	3.0	D.37 ++++
20P	353	3.0	K.37 ++++
24B	406	1.25	D.49 ++++
24P	406	1.25	K.50 ++
31P	465	1.25	K.62 ++
31B	465	1.25	K.62 ++
36B	513	1.25	K.57 +++
36B	513	1.25	L.201—good condition
36P	513	1.25	K.57 ++++
36P	513	1.25	L.201—poor condition
40B	546	1.25	L.166—good condition
40B	546	1.25	K.61 ++
40P	546	1.25	K.61 ++++
40P	546	1.25	L.166—poor condition
41B	559	5.0	L.151—good condition
41B	559	5.0	K.63 +++
41P	559	5.0	L.151—good condition
41P	559	5.0	K.63 ++++

B=Bile salt medium strain; P=Plain medium strain
L=live; D=dead; K=killed
+, ++, +++, ++++=extent of lesions

animals inoculated with strains of the bacillus from the 20th generation to the 41st generation inclusive, and Group II comprising animals inoculated with strains from the 42nd to the 50th generation inclusive. Tables 1 and 2 show for each group the number of animals inoculated, the generations tested, the dosages employed, the time the strains had been maintained on culture media and the fate of the animals.

TABLE 2

DETAILS OF VIRULENCE TESTS ON RABBITS
GROUP II

Generation	*Period of growth (days)*	*Dose (mg.)*	*Fate of test animals (days)*
42B	572	1.25	K.61 +
42B	572	1.25	L.144—good condition
42P	572	1.25	K.61 ++++
42P	572	1.25	L.144—poor condition
43B	584	1.25	D.16 intercurrent infection
43B	584	1.25	K.61 ++
43P	584	1.25	K.61 ++
43P	584	1.25	L.140—poor condition
44P	596	1.25	K.65 ++++
44P	596	1.25	L.136—good condition
44B	596	1.25	K.65 +
44B	596	1.25	L.136—good condition
45B	605	1.25	K.69 ++
45B	605	1.25	D.24 intercurrent infection
45P	605	1.25	K.69 +++
45P	605	1.25	L.132—poor condition
46P	616	1.25	K.62 ++++
46P	616	1.25	L.93—poor condition
46B	616	1.25	K.62 ++
46B	616	1.25	L.93—good condition
49B	661	1.25	L.84—good condition
49P	661	1.25	L.84—good condition
50P	671	1.25	L.60—good condition
50B	671	1.25	L.60—good condition

B=Bile salt medium strain; P=Plain medium strain
L=live; D=dead; K=killed
+, ++, +++, ++++=extent of lesions

Group I

Rabbits inoculated with plain medium strains. In animals which died or were sacrificed the gross lesions were confined to the lungs and consisted of miliary tubercles distributed throughout the lung tissue but especially beneath the pleura. The tissue response in all rabbits was quite similar and was characterised by the presence of nodular areas comprising aggregations of histiocytes and lymphocytes (Fig. 1). In some cases the lymphocytes were arranged in tightly packed rings around the histiocytes but more frequently no such arrangement was seen. Vacuolation and chromatolysis of the nuclei of the histiocytes was noted in many sections. Giant cells were not numerous. When present, their nuclei were seen to be clumped at one pole of the syncytium, rather than arranged around its periphery.

Some foci showed necrosis and caseation in their centres. Alveolar collapse was very often seen and several alveoli contained numerous phagocytic cells. Acid-fast bacilli were often demonstrated, chiefly in the necrotic foci, although usually not in large numbers.

The three animals of this batch that were left alive were listless, disinclined to eat and in very poor condition.

Rabbits inoculated with bile salt medium strains. The gross lesions resembled those found in the rabbits inoculated with strains grown on plain medium. Histological examination showed a more diffuse type of reaction and nodule formation was less frequent. (Fig. 2). In addition to extensive histiocytic and lymphocytic interstitial infiltration, slight alveolar proliferation and some alveolar phagocytes were observed. Caseation was not seen. Acid-fast bacilli were found in some sections. Three rabbits allowed to live beyond the two-month period were in good condition and feeding well.

Group II

Rabbits inoculated with plain medium strains. The gross and microscopic lesions found in the animals which died or were killed were indistinguishable from those described in respect of the animals in Group I inoculated with strains grown on plain medium. Of the 7 rabbits left alive, three were in very poor condition, listless and disinclined to feed and four were in good order and feeding well.

Rabbits inoculated with bile salt medium strains. Apart from two rabbits which died from intercurrent disease, the lesions observed were very slight and confined to the lungs. They consisted of a few red pin-point foci, found chiefly sub-pleurally. While the rabbits inoculated with earlier generations of the organism on bile salt medium had shown a diffuse cellular interstitial infiltration of the lungs without formation of distinct foci, those which received the later subcultures showed considerably less interstitial reaction and larger areas of normal lung tissue. Furthermore, the infiltrating cells were primarily lymphocytes rather than histiocytes. In these latter animals no congestion was observed but some sub-plural emphysema was found; one animal showed acid-fast bacilli. The last rabbit inoculated showed only a few areas of interstitial infiltration consisting of a few histiocytes surrounded by lymphocytes. No giant cells were seen. (Fig. 3).

There were five remaining rabbits of this batch all of them in good condition and feeding well.

Summarising the post mortem findings for the two groups it will be seen that all 11 rabbits inoculated with the plain medium strains showed more or less extensive lung lesions; in three animals these changes were less progressive in nature than in the remaining eight. Of 13 rabbits inoculated with the bile salt medium strains six, all inoculated with earlier generations, were classified on post-mortem examination as having acute progressive lesions, while seven showed lesions of a milder and more limited kind (Table 3).

TABLE 3

THE GENERAL NATURE OF THE LESIONS FOUND IN RABBITS AT AUTOPSY

Type of lesion	*Rabbits inoculated with plain medium strain Number*	*Rabbits inoculated with bile salt medium strain Number*
"Extensive"	8	6
"Limited"	3	7

Of the 10 rabbits inoculated with the plain medium passaged strains and allowed to live on, six were in poor condition, listless and not feeding, while four were in good condition and feeding normally. In contrast, all eight rabbits inoculated with the bile salt medium strains and allowed to live were in good condition and feeding well.

DISCUSSION

Various claims as to the number of passages and length of time on bile salt media necessary for the production of attenuated strains of *Mycobacterium tuberculosis* have been made. Calmette and Guérin in the early stages of their investigations, considered 42 passages sufficient but eventually 230 subcultivations were required to render B.C.G. satisfactorily avirulent. In the present work, virulence tests with passaged strains were not commenced until the 20th subculture was reached since it was considered that no appreciable attenuation would have taken place up to this point.

The determination of virulence of an organism is complicated by the interaction of a number of factors extrinisic to the organism itself. Important among these are varying degrees of susceptibility of the experimental animals to the test organism influenced perhaps by varying effects of environmental factors, and variations in technical procedure. In this series of experiments great care was taken to render the procedure as uniform as possible throughout. The animals were from one stock and were subjected to the same constant environment throughout the experiments.

From the results obtained it seems clear that the method described was capable of detecting differences in virulence between the two strains under test and between different generations of the bile salt medium strain. However, a review of the nature and extent of the lesions produced suggests that more critical results probably would be obtained by using smaller test doses of vole bacillus intravenously and by not sacrificing the rabbits for examination until at least three to four months after inoculation. In the present instance, circumstances did not permit of this course being taken. The reduction in extent of the lesions in animals inoculated with the later bile salt medium generations, from the 44th subculture onwards,

suggests some diminution in virulence of the organism. Also, the nature of the lesions supports this suggestion.

The significance of the cytological structure of lesions as an index of virulence of the infecting strain of *Mycobact tuberculosis* was discussed by Oatway and Steenken (1936) in dealing with differences in pathogenicity between virulent and avirulent strains of the tubercle bacillus. The mononuclear cell was held by these authors to form the basis of the tubercle reaction, becoming in older lesions the epithelioid cell or "sick" monocyte. A preponderance of such cells was taken by these authors to indicate virulence on the part of the infecting strain, whereas the presence of large numbers of lymphocytes, particularly when arranged in a wide, tightly packed peripheral zone around a nodule, was taken as evidence of infection by a relatively avirulent organism. These findings were supported in Oatway and Steenken's experiments by other criteria of virulence and the consistency with which the relevant cytological reactions were produced by virulent and avirulent strains rendered such findings reliable as a guide to the pathogenicity of the infecting organism. Although this work was done with avian and human type strains, it does not appear unreasonable to apply the findings in an appreciation of the results of the present work with the vole bacillus. When this is done, it is notable that the only rabbits showing the milder type of lymphocytic interstitial tissue response were those included in Group II inoculated with the later generations of the strain grown on bile salt medium. However, it must be pointed out that statistical analysis of the data contained in Table 3 concerning the nature and extent of the lesions found at autopsy does not indicate a significant difference between the numbers of "extensive" and "limited" lesions found in the rabbits inoculated with the plain medium strain and those found in the rabbits inoculated with the bile salt medium strain.

The condition of all animals remaining alive at the close of the experiment is summarised in Table 4. The observations on which the animals were classified were made from 201 days after inoculation in the case of the 36th generation to 60 days after inoculation in the case of the 50th generation. When subjected to statistical analysis,

TABLE 4

THE GENERAL CONDITION OF RABBITS NOT SACRIFICED AFTER INOCULATION

Condition of animal	*Rabbits inoculated with plain medium strain*		*Rabbits inoculated with bile salt medium strain*	
	Number	*Per cent*	*Number*	*Per cent*
Good	4	40	8	100
Poor	6	60	0	0

the difference in numbers of rabbits in "poor" and "good" condition between the plain medium group and the bile salt medium group is found to be significant. The standard deviation is 22.47 per cent and the observed difference is 60 per cent, giving a standard error of 2.66. Furthermore, the value for χ^2 is 7.2 giving a P (probability) figure of less than 0.01.

It is apparent that while the earlier post mortem findings revealed no marked difference in virulence between the two passaged strains, the results of post mortem examinations made on animals which had been inoculated with later subcultures, from the 44th onwards, suggested some diminution in virulence of the bile salt medium passaged strain. The marked difference in condition between animals not sacrificed after inoculation with plain medium passaged strains and those not sacrificed after inoculation with bile salt medium passaged strains, in favour of the latter, more strongly supports the view that passage on the bile salt medium resulted in reduction in virulence. No alteration in virulence of the strain subcultivated on plain medium was detectable. It is noteworthy that even in the early subcultures, from the 36th onwards, the bile salt medium strain seemed to be less pathogenic than the plain medium strain when judged by its effects on rabbits observed over a period of time. The degree of attenuation produced, therefore, is best demonstrated when the inoculated animals are allowed to live for at least four months. Further work is required to determine the antigenic value of an attenuated strain of the vole bacillus.

SUMMARY

A method for the attenuation of strain LV 2401 of the murine type of tubercle bacillus by repeated and rapid passage on culture medium containing bile salt is described and the cultural changes produced in the organism during subcultivation are recorded.

Pathogenicity tests on rabbits have been conducted with two strains of the original LV 2401 culture, one passaged on plain medium and the other on bile salt medium. It is suggested that the results of these pathogenicity tests indicate some attenuation of the vole bacillus strain cultivated on bile salt medium and no attenuation of the strain cultivated on plain medium.

ACKNOWLEDGMENTS

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LEGENDS FOR FIGURES

Fig. 1. Section of lung of rabbit No. 79. A nodule is shown with giant cells and numerous histiocytes. Lymphocytes are not very numerous.
Stained haematoxylin and eosin ×200

Fig. 2. Section of lung of rabbit No. 80. A diffuse interstitial lymphocytic and histiocytic infiltration is shown. Giant cells are present.
Stained haematoxylin and eosin ×200

Fig. 3. Section of lung of rabbit No. 116. Relatively slight changes are present. A small focus is shown and empysema is visible. The interstitial infiltration is primarily lymphocytic in character.
Stained haematoxylin and eosin ×200

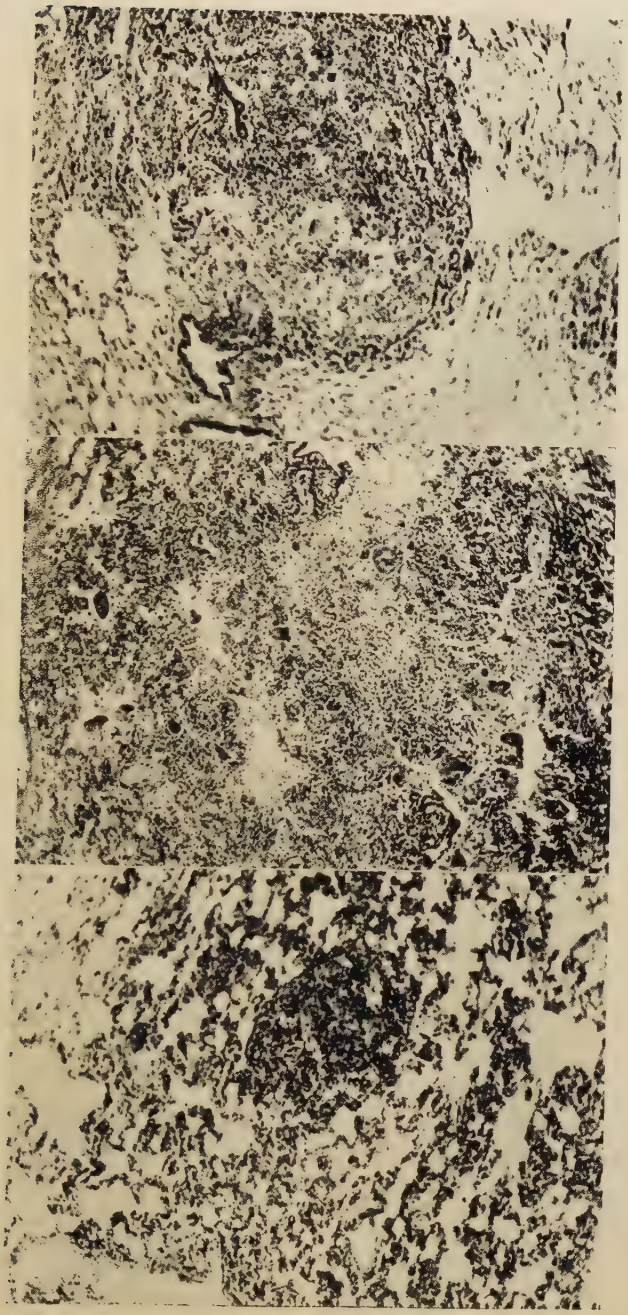

Fig. 1.

Fig. 2.

Fig. 3.

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STUDIES OF THE TOXICITY OF BRACKEN
(*Pteris Aquilina*)

By

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INTRODUCTION

The investigations recorded here were done to study the influence of bracken feeding upon the thiamin content of the rumen and to explore the possibility of the presence of another toxic factor. Bracken poisoning in cattle can neither be prevented nor cured by injecting massive doses of thiamin. The literature on this subject has been discussed elsewhere (Shearer, 1945; Evans and Evans, 1949; Evans, Evans and Roberts, 1951; Naftalin and Cushnie, 1954) and recapitulation is unnecessary.

The plan of the experiments on cattle has been reported by Naftalin and Cushnie (1954), who give a detailed account of the pathology of bracken poisoning. The animals that were cannulated in the rumen served as donors of rumen contents for the experiments described here.

Additional information on the pyruvate concentration of the blood of four bracken-fed heifers and four control animals is included, together with other data of the experimental animals.

METHODS

Before a study of the thiamin content of the rumen digesta of bracken-fed animals could be undertaken an investigation into the best way of supplementing the diet of thiamin-deficient rats with rumen contents was necessary. The work of McElroy and Goss (1941) showed that the response to such supplementation was very variable when dried whole rumen contents were used. Thiamin is unstable when heated under alkaline conditions but is not destroyed by strong mineral acids. We have found that drying rumen contents without taking the precaution of reducing the pH below 4 leads to considerable loss of thiamin which probably explains the erratic results of McElroy and Goss. At the time the experiment described here was done, oven drying of whole rumen contents had been found to be unsatisfactory and a procedure was adopted designed to remove much of the coarse fibrous material and to concentrate the micro-organisms in which thiamin would presumably be less exposed to the thiaminase of bracken than any thiamin in solution.

Preparation of rumen "sludge". Rumen contents were withdrawn by suction through a rumen cannula using an ebonite tube which was thrust well into the liquid material in the ventral sac. The contents so obtained were strained through four layers of surgical gauze and the liquor was then spun in a Sharples centrifuge. The deposit so obtained (henceforth called "sludge"), which was rich in micro-organisms, was used to supplement the ration of thiamin-deficient Hooded weanling rats of the Rowett Institute stock.

In later experiments the sludge was acidified in between pH 1 and 2 with sulphuric acid and boiled for fifteen minutes in order to destroy any

thiaminase present—which is heat labile (Evans and Evans, 1949)—whilst preserving the thiamin. The solid material was then separated again in a Sharples centrifuge and the supernatent was concentrated by heat to a sticky mass and added to the sludge. This latter operation was necessary as the thiamin contained in the sludge largely passed into the supernatent phase.

The comparison of the thiamin content of rumen sludge from bracken- and hay-fed heifers. Single litters of eight weanling rats, containing four rats of each sex, were used to compare the sludge obtained from bracken- and hay-fed heifers. The animals were placed on a thiamin-deficient basal ration—see Ration A in Table 1—one week after weaning and daily weights were recorded. The ration was supplemented by rumen sludge when the rats started losing weight and the sludge was added so that it formed approximately 12 per cent of the ration on a dry matter basis. This rate of supplementation was determined by an experiment, the results of which are given in Fig. 1.

FIGURE 1

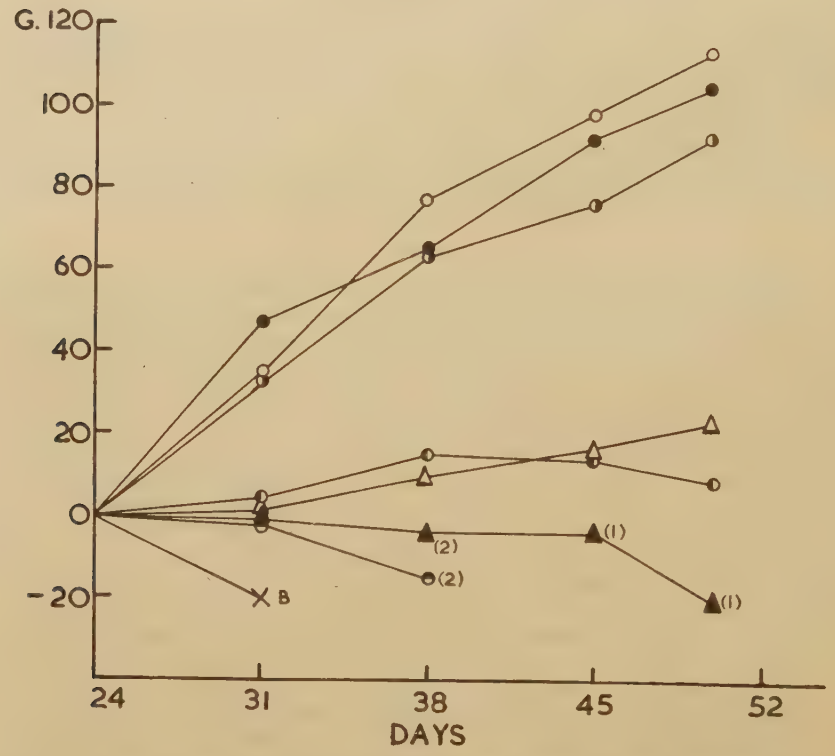

Fig. 1. The weight changes of thiamin-deficient rats following supplementation with ●——● 60 μg. thiamin or ◐——◐ 10 ug. thiamin per 100 g. ration; ○——○ 16%, ◑——◑ 8% (one male and one female) or ◓——◓ 4% fresh rumen sludge; △——△ 16% or ▲——▲ 4% oven-dried rumen sludge or ——x with no supplementation. Each line represents the mean of two male and two female rats from litters randomised throughout the groups with one exception which is indicated above. Figures in brackets show the number of survivors at that stage and the line subsequent to these figures represents this numbers of rats.

The comparison of rumen sludge from bracken- and hay-fed heifers in thiamin-adequate rats. Sixteen males from four litters were used for experiments in which large supplements of rumen sludge were given to thiamin-sufficient rats. Litters were evenly distributed throughout the experimental groups. Ration B was used for these experiments as given in Table 1. Rumen sludge was added to form approximately 30 per cent of the ration on a dry matter basis; drinking water was replaced by rumen liquor, but as the fluid intake was then sometimes excessive additional water was provided when necessary. Food and liquor intakes were recorded. Thiamin was injected subcutaneously three times weekly at the rate of 210 μg. per week.

TABLE 1

COMPOSITION (%) OF THE THIAMIN-FREE RATION A AND OF THE YEAST RATION B

The vitamin supplement added to Diet A is given as mg. per 100 g. ration.

Diet A		*Diet B*	
Casein (Genatosen; low vitamin content)	20	Casein (Glaxo C)	20
Lard	14	Yeast	8
Sucrose	60	Lard	10
McCollum's Salts (B.P.) with iron	5	Corn starch	18
Cod liver oil	1	Sucrose	10
Vitamin supplement		McCollum's Salts (B.P.) with iron	3
Pyridoxinehydrochloride	0.3	Cod liver oil	1
Riboflavine	0.3	Choline chloride	(22 mg./70 g. diet)
Calcium pantothenate	2.0	Wheat germ oil	75 mg. daily
Nicotinic acid	4.0		
l-inositol	10.0		
Para-aminobenzoic acid	10.0		
Wheat germ oil	75 mg. daily		

The management of cattle. Two heifers each with a rumen cannula were used to provide rumen contents. One of them was fed sun-dried bracken of the same harvest that had produced bracken poisoning in the previous year (1950) when fed at the rate of 60 per cent of the ration, the remainder consisting of hay; the other heifer was fed hay alone. As no signs of bracken poisoning in the bracken-fed heifer had occurred after 66 days, the dried bracken and hay ration was replaced by 100 per cent fresh green bracken (Naftalin and Cushnie, 1954).

The animals in the previous year (1950) had received 60 per cent sun-dried bracken and 40 per cent hay, or 60 per cent sun-dried bracken, 20 per cent molasses and 20 per cent hay. All these animals had died of bracken poisoning. Jugular blood was drawn towards the end of the feeding period at regular intervals from four of them and from four hay-fed control heifers in order to determine the pyruvate concentration. The method used was that of Friedmann and Haugen (1943).

RESULTS

Preliminary

The result of the preliminary experiment which was done to determine the quantity of rumen sludge needed to produce adequate growth in thiamin-deficient rats is given in Fig. 1. Comparisons

were made between fresh and oven-dried (24 hours at 80°C.) rumen sludge at three levels, namely to form 4, 8 and 16 per cent of the ration on a dry weight basis. The responses obtained were compared to those obtained by adding 10 and 60 μg. thiamin per 100 g. basal ration of the rats, or by the basal ration alone.

It can be seen from Fig. 1 that the response to 60 μg. thiamin per 100 g. ration was very similar to that of using the 16 or 8 per cent level, while the response of the 4 per cent level of fresh sludge was rather less than that obtained from 10 μg. per cent of thiamin. The oven-dried sludge clearly had lost a large part of its thiamin content as feeding at the 16 per cent level produced a response approximately equal to 10 μg. per cent thiamin. The sludge fed

FIGURE 2

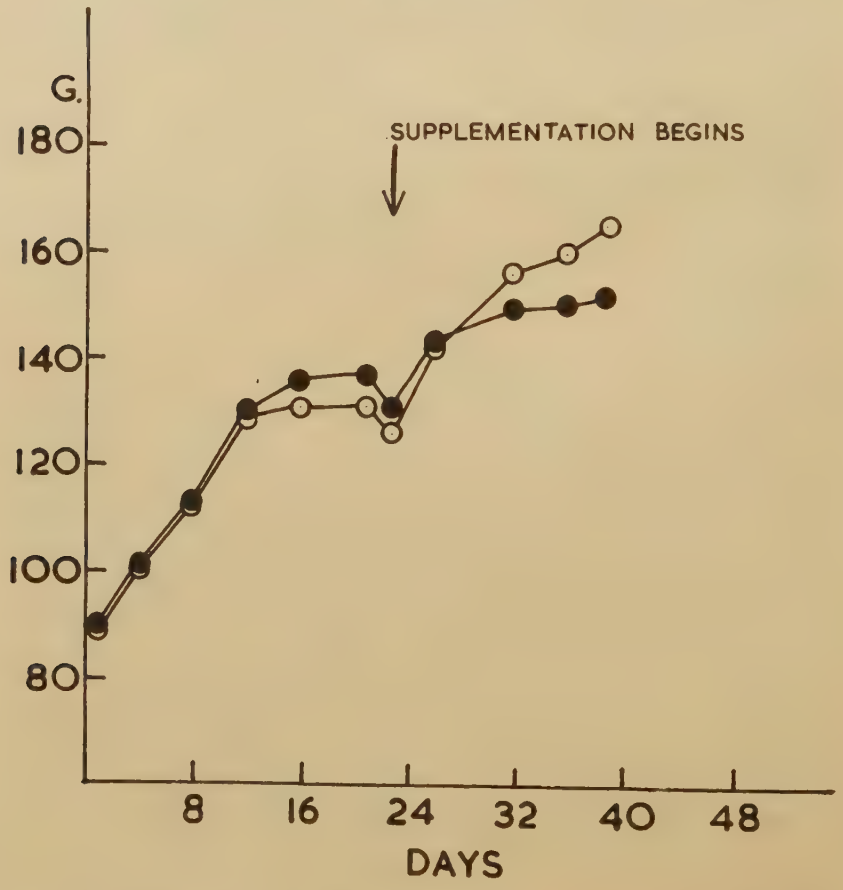

Fig. 2. A comparison between fresh, O——O, and freeze-dried, ●——●, rumen sludge fed at the 8% level to the thiamin-deficient rats. The arrow indicates the beginning of supplementation. Each line represents the mean of four males and four females from two litters equally distributed between the groups.

at the 4 per cent level both in the fresh and dry states was inadequate to allow any growth. This is curious as it represents one-quarter of the 16 per cent level which, with the fresh sludge, produced a similar response to 60 μg. per cent thiamin, while 10 μg. per cent thiamin, one-sixth of the higher supplementation, produced a better result than 4 per cent fresh sludge. This is possibly due to the unpleasant smell of rumen sludge which with rats in a poor state of thiamin adequacy may be sufficient to stop them eating.

As the preparation of rumen sludge is time-consuming and as drying was ineffective in preserving the thiamin content, a trial with sludge in the fresh state compared to sludge after freeze drying was undertaken. Sludge was added to form 8 per cent of the dry matter of the ration. The results are given in Fig. 2 showing that the freeze-dried material tended to be inferior to the fresh material, the difference approaching significance at the 5 per cent level. The results were not regular as four rats fed freeze-dried rumen sludge made weight gains of less than 10 g. while the remaining four

FIGURE 3

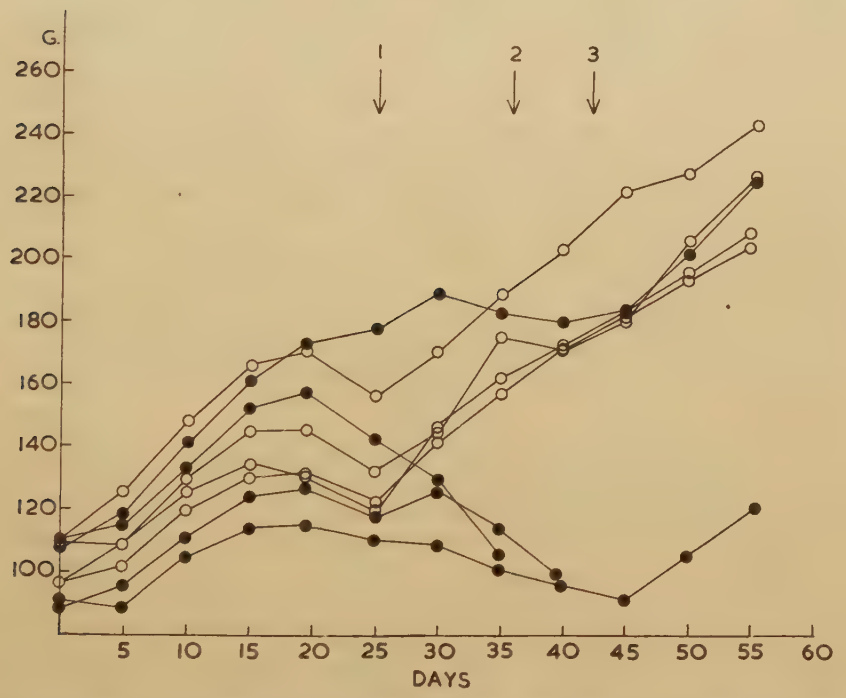

Fig. 3. Individual weight curves for four male and four female rats from one litter during thiamin depletion and following supplementation with the sludge of rumen liquor incubated with dried grass O——O, or dried bracken ●——●. The arrows indicate: (1) supplementation with untreated sludge; (2) supplementation with sludge boiled at an acid pH and neutralised; and (3) reversal of the groups.

made weight gains that were comparable to those of rats fed fresh rumen sludge.

There was no alternative to the use of the fresh material and this was prepared daily in all subsequent experiments, stored at -1° C., and fed within 24 to 48 hours of preparation. In most experiments it was used to supplement thiamin-deficient rations at the rate of 12 per cent of the total dry matter. This level was chosen as it was half-way between the 8 and 16 per cent levels which fell on either side of the curve produced by 60 μg. of thiamin per 100 g. ration (Fig. 1).

The Effect of Incubating Rumen Liquor with Bracken or Dried Grass

The possibility that the micro-organisms of the rumen would destroy the thiaminase of bracken was tested by incubating ground dried bracken, or ground dried grass as a control, with rumen liquor. 50 g. of each material was incubated with 1200 ml. of freshly drawn rumen liquor under an atmosphere of carbon dioxide for 4 hours at 37°C. The sludge was then spun off, collected and used to supplement the ration of thiamin-deficient rats (4 to a group) at the rate of 16 per cent of the dry matter of the ration. As those receiving bracken sludge made no sustained response both sludge preparations were boiled, after being acidified, for fifteen minutes. After a further period of a week the bracken rats still made no response and so the groups were reversed. After a lag of three days the two remaining rats in the bracken-sludge group responded to the grass sludge. The results are summarised in Fig. 3. This experiment showed that the micro-organisms of the rumen did not readily destroy the thiaminase.

A Comparison of the Thiamin Content of the Rumen Sludge of a Bracken- and a Hay-fed Heifer

Six experiments were done and Table 2 shows the time relation between the feeding of bracken to the experimental heifer and the rat experiments. It can be seen that the whole period between feeding

TABLE 2

THE RELATIONSHIP BETWEEN THE BRACKEN FED TO A HEIFER AND THE EXPERIMENTS CONDUCTED ON THE THIAMIN-DEFICIENT RATS FED THE RUMEN SLUDGE OF THIS HEIFER

No. of experiment	*Diet of heifer*	*Beginning of depletion*	*Sludge supplement fed*	*End of experiment*
1	60% sun-dried bracken plus 40% hay	25th April	22nd May	6th June
2	do.	8th May	6th June	23rd June
3	do.	18th „	16th „	29th „
4	do.	1st June	29th „	3rd August
5	do. changed to 100% fresh green bracken on July 6th	14th „	11th July	13th „
6	Fresh green bracken. Heifer died Sept. 20th	20th July	14th August	1st Sept.

sun-dried bracken—one-year-old—and the death of the heifer after the ration was changed to fresh green bracken is covered by the six experiments with rats, in all of which sludge was fed at the rate of 12 per cent of the dry matter of the ration.

Experiments 1, 2 and 3. Fresh sludge, untreated by heat, was used and a clear difference between the two groups of rats occurred. The second experiment is shown in Fig. 4 and the rats received no benefit from the fresh bracken sludge. Two rats on the bracken sludge were injected with thiamin towards the end of this experiment

FIGURE 4

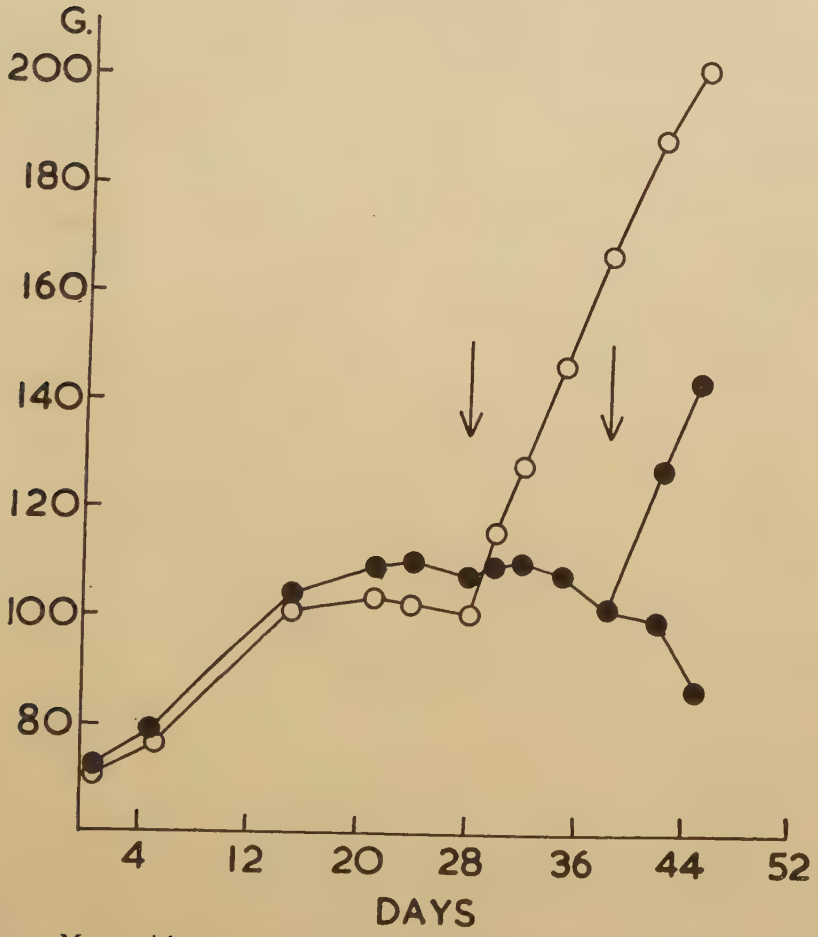

Fig. 4. Mean weight curve representing four rats, two male and two female from one litter, during thiamin depletion and following (arrow 1) supplementation with rumen sludge from a hay-fed heifer O——O, or rumen sludge from a bracken-fed heifer ●——●. The second arrow indicates that thiamin given as three subcutaneous injections at the rate of 120 μg. per week was administered to two rats (one of each sex) of the bracken group.

at the rate of 120 μg./week and the immediate response indicated that lack of thiamin was the limiting factor to growth. The third experiment gave a similar result. These experiments show either that little or no thiamin was present in the bracken sludge or that thiaminase was present.

Experiment 4. In this experiment the sludge from both heifers was boiled for 15 minutes at a pH between 1 and 2 in order to destroy any thiaminase present in the bracken sludge. The sludge was then spun off and used to supplement the deficient rats. After one week neither group had gained weight. It was probable that boiling the sludge had liberated intracellular thiamin into the supernatent fluid which was discarded. The procedure was, therefore, changed so that the supernatent fluid was concentrated by heat at an acid

FIGURE 5

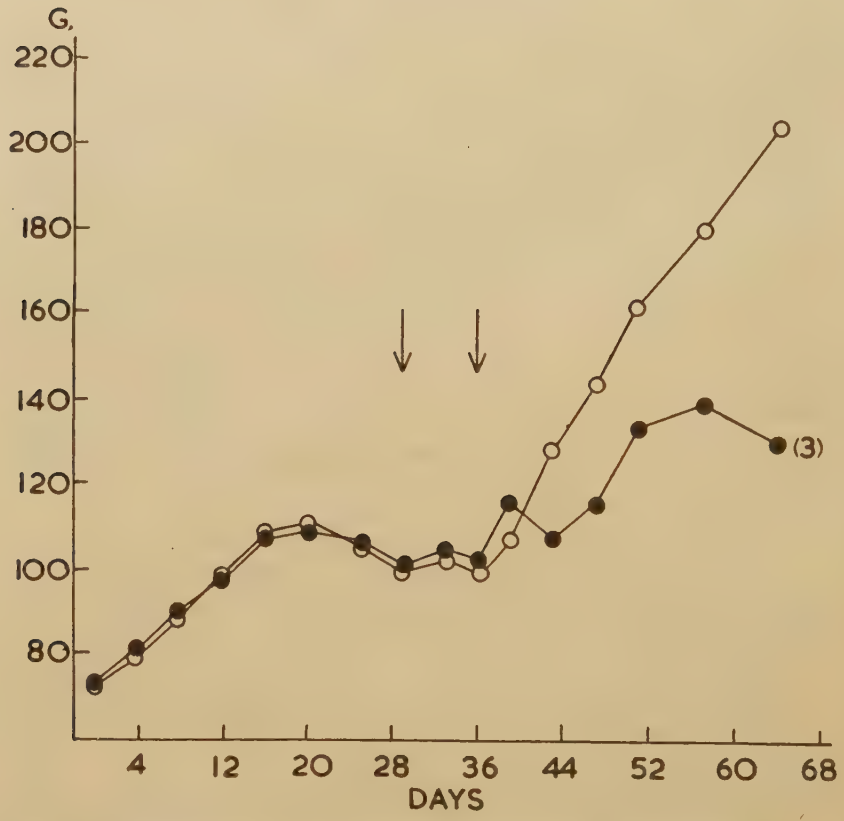

Fig. 5. The mean growth rate of four rats, two of each sex, from one litter during thiamin depletion and following supplementation of rumen sludge from a hay-fed heifer O——O, or a bracken-fed heifer ●——●. Arrow 1 indicates supplementation with acid boiled sludge without supernatent fluid. Arrow 2 indicates the addition of the concentrated supernatent to the sludge. The number in brackets indicates the number of survivals.

pH and added to the boiled sludge. The material was neutralised before feeding. When this material was substituted for the boiled sludge alone the rats receiving the material from the hay-fed heifer put on normal weight gains while after a lag the rats receiving the bracken sludge followed suit. The weight gains of the latter group, however, were not maintained and ultimately weight losses occurred. The course of this experiment is shown in Fig. 5. The results of the previous three experiments were therefore judged to be due largely to the presence of thiaminase in the "bracken" sludge fed to the rats although even when this was destroyed, insufficient thiamin was left to provide for normal growth in the depleted rats.

FIGURE 6

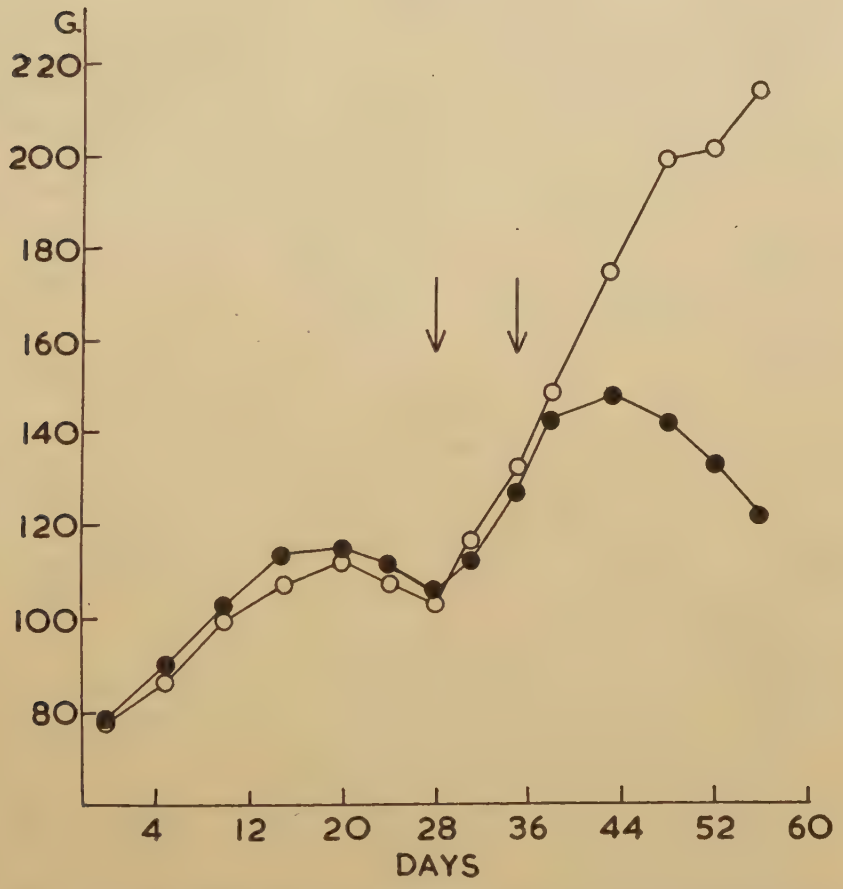

Fig. 6. The mean growth rate of four rats, two of each sex, from one litter during thiamin depletion and following supplementation (arrow 1) with acid boiled rumen sludge with added supernatent fluid from a hay-fed heifer O——O, or a bracken-fed heifer ●——●. Arrow 2 indicates the change of ration of the bracken-fed heifer from sun-dried to fresh green bracken.

Experiment 5. The preparation adopted in the second period of experiment 4 was used in both experiments 5 and 6. During experiment 5 the ration of the heifer receiving bracken was changed so that it consisted entirely of fresh green bracken (Table 2). This was soon reflected in the growth rate of the rats receiving boiled bracken sludge as all rats ceased to gain weight after a short lag. The experiment is depicted in Fig. 6.

FIGURE 7

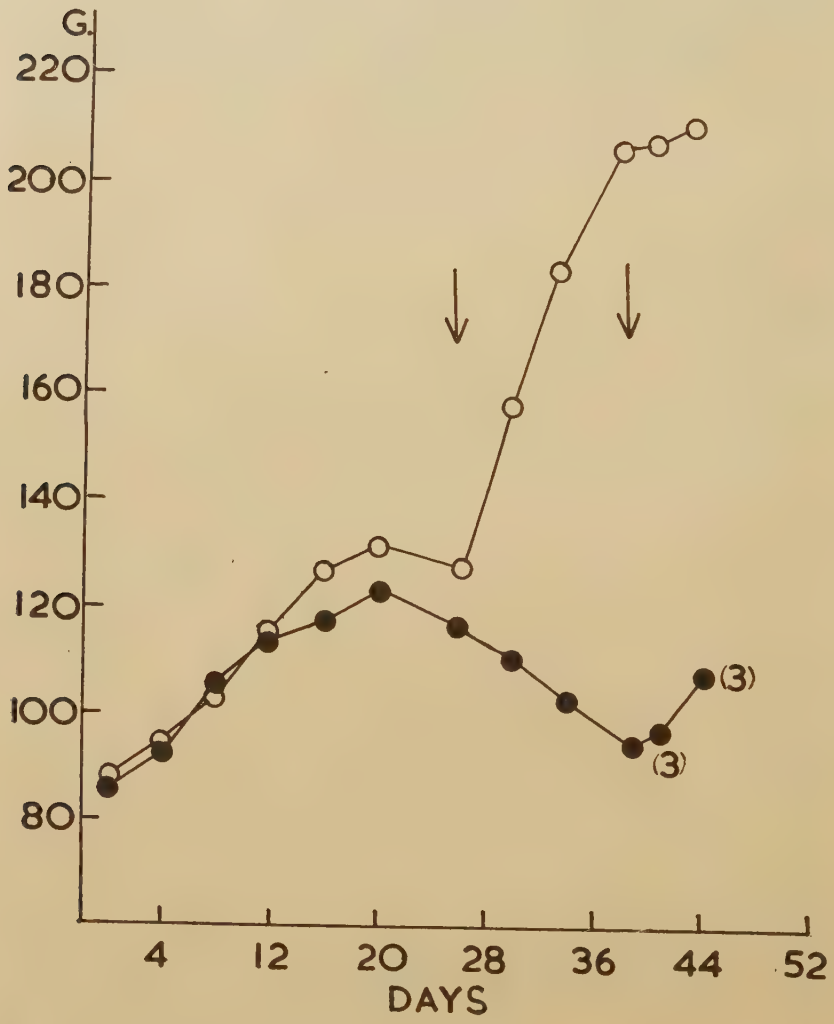

Fig. 7. The mean growth rate of four rats, two male and two female from one litter, during thiamin depletion and following supplementation (arrow 1) with rumen sludge (acid boiled plus supernatent) from a hay-fed heifer O——O, or a green bracken-fed heifer ●——●. Arrow 2 indicates reversal of the group, the figures in brackets the number of survivors.

Experiment 6. This experiment gave the same result as experiment 5 and showed that the neutralised, acid boiled sludge with the supernatent fluid added from the bracken-fed heifer did not contain sufficient thiamin to allow growth of the rats, while those receiving the same quantity of sludge treated in exactly the same way from the hay-fed heifer grew normally. At the end of this experiment the groups were reversed and a response was immediately seen in the rats previously receiving bracken sludge. The experiment is shown in Fig. 7.

The Toxicity of Bracken to Rats

Rats fed a diet containing 40 per cent dried bracken attain normal growth if they are given adequate thiamin by injection (Evans and Evans, 1949; Carpenter, Phillipson and Thomson, 1950). Rats, however, which receive an adequate basal ration with 60 per cent dried bracken make very small weight gains if given adequate thiamin with and without other B vitamins by injection, as is shown in Fig. 8. This compares well with the finding of Cordy

FIGURE 8

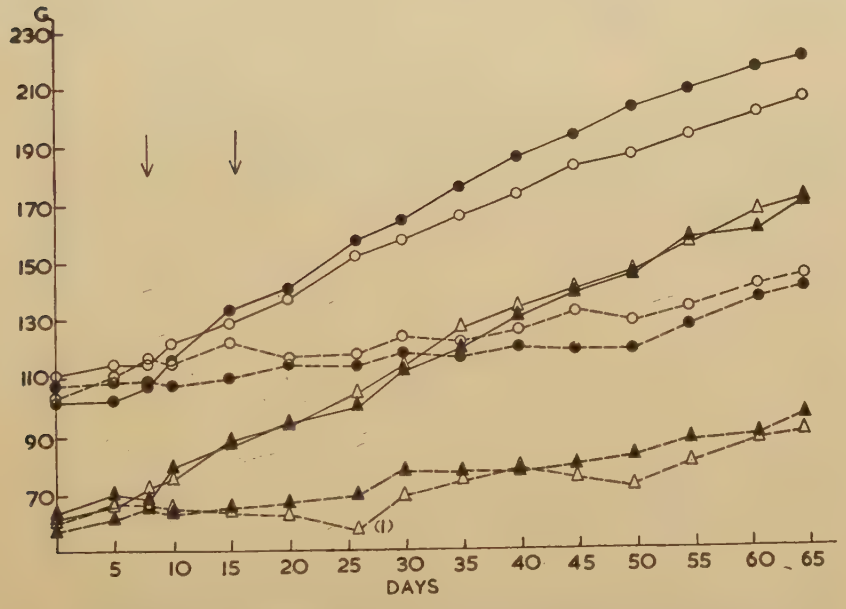

Fig. 8. The mean growth rates of two rats fed basal ration (Table 1, Diet B) plus 40% (unbroken line) or 60% (broken line) dried bracken. The symbols indicate: O male and △ female rats receiving thiamin by subcutaneous injection at the rate of 120 μg. per week in three injections; ● male rats and ▲ female rats receiving weekly thiamin 120 μg; riboflavine 300 μg; pyridoxine hydrochloride 300 μg.; calcium pantothenate 2000 μg; nicotinic acid 4000 μg; para-aminobenzoic acid 10,000 μg. and *l*-inositol 10,000 μg. by three injections.
All rats received 40% bracken until the 8th day (Arrow 1); half the groups received 50% bracken until the 15th day (arrow 2) and thereafter 60% bracken. The figure (1) indicates death of one of the rats of this group.

(1952) who noted only small weight increases in rats fed a ration containing 50 per cent autoclaved bracken. To see whether this is indicative of any other material which is detrimental, two experiments were done in which the basal ration of normal rats receiving thiamin by injection (see Diet B, Table 1) was supplemented by rumen sludge from a bracken-fed or grass-fed heifer at the rate of 30 per cent of the ration on a dry matter basis. The donor animals were the two heifers fed bracken during 1952 (Table 4); one was fed green bracken and the other cut grass. The first bracken-fed animal became acutely ill, showing typical signs of bracken poisoning but ultimately recovered when fresh grass was provided. The other animal was then fed bracken and ultimately died. The sludge was either boiled or left unboiled but without acidification, and the supernatent was concentrated by heat as in the previous experiments and added to the sludge. Food consumption and weight gains were recorded and drinking water was replaced by rumen liquor from the experimental heifers and the fluid intake was measured. As the rats consumed considerable quantities of rumen liquor additional water was allowed when the drinking pots were dry. The weight increases, food consumption, and fluid intake in the two experiments are given in Table 3.

TABLE 3

WEIGHT GAINS, FOOD INTAKE (g.) AND FLUID CONSUMPTION (ml.) OF RATS FED ON DIET B SUPPLEMENTED BY 30% RUMEN SLUDGE ON A DRY MATTER BASIS. EACH GROUP COMPRISED 4 MALE RATS TAKEN FROM FOUR LITTERS AND DISTRIBUTED EVENLY THROUGH THE 4 GROUPS

Group	*Supplement*	*Mean wt. gains*	*Mean food consumption*	*Food fed to produce 1 g. wt. increase*	*Water consumption*
Experiment 1	(15 days' duration)				
1	Hay sludge	33.7	189	5.6	568
2	Boiled hay sludge ..	25.5	146	5.7	516
3	Bracken sludge†	29.2	183	6.2	484
4	Boiled bracken sludge ..	22.1	164	7.4	591
Experiment 2	(25 days' duration)				
1	Hay sludge	115	547	4.8	778*
2	Boiled hay sludge ..	96.9	482	5.0	869
3	Bracken sludge	89.1	453	5.2	986
4	Boiled bracken sludge ..	73.2	398	5.8	1149

†One rat died before the end of the experiment and these figures relate to the remaining 3 rats.

*Rumen liquor plus water.

In both experiments the group which grew best was the group supplemented by hay sludge and the group which made least weight gains was the group fed on boiled bracken sludge. The trends in the ratios between weight gains in relation to the quantity of food eaten is the same in both experiments. Those fed on grass

sludge whether boiled or unboiled ate less food per g. weight increase than those fed on bracken sludge. This is most marked in the first experiment. After calculating the quantity of food eaten in excess of basal requirements—using the data given by Brody (1945) to obtain approximate values for the basal requirements of rats of different weights—and determining the number of calories used in producing a gram increase in weight, it appears that there is little difference between the four diets excepting that in both experiments one rat receiving boiled bracken sludge required more calories per g. increase in bodyweight than those of the remaining three groups. The calculated values for each rat are given in Table 4.

TABLE 4

CALCULATED VALUES FOR THE CALORIES USED BY EACH RAT FED RUMEN SLUDGE AT THE 30% LEVEL TO INCREASE ITS WEIGHT BY ONE GRAM

Group	*Supplement*	*Cals./g.*			
Experiment 1					
1	Hay sludge	12	11	10	9
2	Boiled hay sludge ..	9	7	8	6
3	Bracken sludge	—	11	10	10
4	Boiled bracken sludge ..	12	19	11	12
Experiment 2					
1	Hay sludge	12	11	10	12
2	Boiled hay sludge ..	10	13	11	10
3	Bracken sludge ..	12	12	10	10
4	Boiled bracken sludge ..	10	10	10	15

The large consumption of fluid in these experiments, of which the larger part was rumen fluid, boiled or unboiled, is probably the result of the high potassium content of rumen fluid; even so, the quantities consumed by the rats receiving bracken sludge and bracken liquor are greater, particularly with group 4, than with those receiving their fluid and rumen supplement from the grass-fed heifer. One death occurred in group 3 of the first experiment and the figures given are for the remaining three rats; otherwise no rat showed any signs of any syndrome resembling bracken poisoning.

The Mortality Rate of Bracken-fed Heifers

Investigations on bracken poisoning in the Institute from 1949 to 1952 have involved feeding fresh green bracken or bracken dried in different ways to fifteen heifers. Of these only one has failed to show clinical signs of poisoning, i.e. haematuria, elevated rectal temperature, petechial haemorrhages, etc. and only one has recovered from poisoning. The details are given in Table 5.

The clinical syndrome is well known and bears little resemblance to classical thiamin deficiency. In the 1950 experiment several of the animals were examined for signs of locomotor incoordination such as can be recognised in horses suffering from bracken poisoning, but none were found. Cardiac irregularities, however, were observed in several animals but these occurred after the onset of

TABLE 5
FATE OF FIFTEEN HEIFERS FED BRACKEN PREPARED IN VARIOUS WAYS

Year	*No. fed bracken*	*No. dying of bracken poisoning*	*No. of recoveries*	*Proportion of ration made up of bracken*	*Mode of preparation of bracken*
1949	2	1	0	60%	Dried in grass dryer
1950	8	8	0	60%	Cut and sun-dried the same season
				60%	Material prepared in previous season fed for 66 days with no result, then fresh cut green bracken fed
1951	3	3	0	100%	
				100%	
1952	2	1	1	100%	Fresh cut green bracken

haemorrhage and could be explained by haemorrhage into the myocardium causing pressure on the auriculo-ventricular bundle.

Table 6 shows the pyruvate concentration in the blood of four bracken-fed heifers in the 1950 experiment and comparison of the values with those found in control heifers fed on dried grass and hay. A significant rise in blood pyruvate did not occur until 24 to 48 hours before death.

TABLE 6
PYRUVATE (mg./100 ml. BLOOD) CONCENTRATIONS IN JUGULAR BLOOD OF BRACKEN-FED AND CONTROL HEIFERS

Date	*Sept.*	9	12	14	15	18	19	20	21	(21 *later*
Control heifers	1	0.8	0.8	1.25	—	0.8	0.8	0.6	—	
	2	0.8	1.1	0.8	—	0.6	0.9	1.0	—	
	3	0.6	0.8	—	—	0.7	0.8	—	0.9	
	4	0.8	0.8	0.8	—	0.7	0.6	0.6	—	
Bracken-fed heifers	5	0.9†	1.1	0.9	—	0.7	1.35	1.4‡	0.5*	
	6	0.9†	1.0	0.9	2.0‡	—	—	—	—	
	7	1.1	1.1†	—	0.9	1.1	2.0‡	—	—	
	8	0.9	1.2	1.3	—	0.8†	0.9	0.8	1.1‡	2.4*

*Sample taken *post mortem*.
†Indicates onset of first visible signs of poisoning, *i.e.* Nos. 5, 6 and 7 haematuria. No. 8 rise in rectal temperature.
‡Death within twenty-four hours of this value.

DISCUSSION

There can be no doubt that the thiaminase of bracken destroys a considerable part of the thiamin content of the rumen. Approximately 60 per cent of the thiamin present in rumen liquor, such as can be drawn by suction from the rumen of sheep, is in solution and about 25 per cent or less is present in the sludge—i.e. in the fraction rich in micro-organisms (Phillipson and Reid, 1953). Of this latter fraction a large part is probably intracellular. It is likely that intracellular thiamin is less exposed to the effect of bracken thiaminase than thiamin in solution, so that a depletion of thiamin in the sludge of rumen liquor strongly suggests that most of the free

thiamin in solution has also disappeared. It is impossible to say from these experiments that no thiamin was present in the sludge of the heifer when fed green bracken: indeed, the tests employed are qualitative rather than quantitative. They have the advantage over chemical or microbiological estimation in that they give a true test of the availability of thiamin to the rat. Taken in this sense the correct interpretation is that the rumen sludge of a hay-fed heifer contained sufficient thiamin to allow some growth of thiamin-deficient rats but not enough to sustain growth, while rumen sludge of a heifer fed green bracken did not contain enough thiamin to allow any growth at all. Rumen micro-organisms are a valuable source of protein and the addition of sludge to the ration is likely to improve the growth rate. However, the fact that rats fed bracken sludge responded immediately to the injection of thiamin indicates that lack of thiamin was the immediate limiting factor to growth. Clearly, bracken-fed heifers obtain far less thiamin than normally-fed heifers, yet we have failed to observe any signs of the thiamin deficiency in the bracken-fed heifer except immediately before death when the level of blood pyruvate rose. Auscultation and electro-cardiograms of the heart showed that abnormalities were similar to the changes which would be expected from heart block, but in any event this was not a constant feature. The fact that bracken poisoning in cattle can neither be prevented nor cured by injecting massive doses of thiamin (Evans, Evans and Hughes, 1951; Naftalin and Cushnie, 1954) proves that the syndrome is not due to lack of thiamin on the part of the animal. We come, therefore, to the curious situation that cattle apparently require very little thiamin. Rats fed on the same thiamin-deficient ration as used in these experiments continue to lose weight after depletion when 1 μg. thiamin is given daily; they make very slight weight gains when 2.5 μg. are given daily and normal weight gains that are nearly identical occur when 7.5 or more μg. are given daily (Phillipson and Reid, 1953). The following calculation is based on the assumption that the rats receiving sludge from the heifer whilst it was eating green bracken received about 1 ug. thiamin daily. When feeding normally these rats eat about 15 g. of basal ration daily and in the supplemented rats 12 per cent of this is represented by rumen sludge, *i.e.* 1.8 g. of sludge dry matter per day. 100 ml. rumen contents drawn by suction contains 5 to 6 g. of sludge dry matter so that 100 ml. contains approximately 3 μg. thiamin in the sludge. If we assume that all thiamin in solution is destroyed by thiaminase and that 100 litres of rumen contents pass from the rumen in 24 hours then approximately 3 mg. of thiamin pass to be liberated in the small intestine. If we assume that the requirements of the rat and the heifer for thiamin are proportionately the same then as a 100 g. rat requires 7.5 μg. thiamin daily, a 200 kg. heifer requires 15 mg. daily. This is a great deal more than the thiamin available in the sludge of the bracken-fed heifer, and signs of thiamin deficiency

should have been apparent, particularly as any thiamin liberated in the small intestine would probably be destroyed by thiaminase. It can only be concluded that the requirements are proportionately a great deal less than those of the rat.

Micro-organisms that require but cannot synthesise thiamin would be unlikely to survive in the rumen if the available thiamin was completely destroyed. This could be expected, particularly with the rumen protozoa which Masson and Oxford (1951) have shown to have an active carbohydrate metabolism. In fact the holotrich ciliates were found to outlive the bracken-fed heifers and were motile in the rumen contents immediately before and after the death of their host; consequently it is unlikely that no thiamin was present in the rumen. Miss M. J. Masson (personal communication) observed that loss of definition and stainability of many of the rumen cocci occurred shortly before death due to bracken poisoning, but it is not clear whether this is due to factors other than a decrease in the food eaten by the host.

The experiments designed to see whether another toxic factor can be incriminated are inconclusive. When 60 per cent bracken is fed in the ration of thiamin-adequate rats the failure to grow can be attributed to partial starvation for bracken cannot be considered a nutritious foodstuff although its nitrogen content is similar to that of dried lucerne (Carpenter, Phillipson and Thomson, 1950). Besides a decrease in the quantity of circulating platelets Cordy (1952) found that alopecia developed in rats with 50 per cent bracken in their ration and this was not corrected by the injection of thiamin or of thiamin plus pteroylglutamic acid and nicotinic acid although otherwise the rats returned to normality. The only indication from these experiments that a factor distinct from the thiaminase may be concerned is the fact that the sludge from the bracken-fed heifer was not eaten so readily as sludge from the grass-fed heifer, while in two rats boiled bracken sludge was apparently less nutritious than unboiled bracken sludge or hay sludge. It is worth while pointing out that it takes at least 5 weeks to produce bracken poisoning in cattle receiving 60 per cent bracken in their food while these experiments did not exceed 25 days and then only 30 per cent of the ration came from the rumen. The work of Cordy (1952) and the experiments recorded here show that the thiaminase present in bracken is responsible for the principal effects observed in rats but they do not exclude the possibility of another toxic factor which has still to be appreciated.

SUMMARY

Rumen sludge from normally fed heifers when fed at the rate of 8, 12 or 16 per cent of the dry matter of a thiamin-deficient basal ration contains enough thiamin to allow weight gains to occur which are optimal or only slightly suboptimal.

Sludge obtained from a bracken-fed heifer did not contain enough thiamin to allow weight gains to occur in thiamin-deficient rats when fed at the rate of 12 per cent on a dry matter basis.

Thiamin-adequate rats grow normally even when dried bracken forms 40 per cent of their ration but make only small weight gains when bracken forms 60 per cent of their ration.

Rumen sludge from a bracken-fed heifer was less palatable to rats than rumen sludge from a grass-fed heifer.

No clear signs of thiamin deficiency were observed in bracken-fed heifers that ultimately died of bracken poisoning.

ACKNOWLEDGMENT

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OCULAR ASSOCIATIONS OF DAPPLING IN THE COAT COLOUR OF DOGS

II. HISTOLOGY

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INTRODUCTION

Clinical and genetic observations on the ocular anomalies associated with coat colour in various breeds of dog have been recorded by Sorsby and Davey (1954). In the dachshund, collies and other breeds, a dapple coat colour is associated with various minor ocular anomalies of which the complete or partial absence of the tapetum lucidum is constant. Breeders do not mate dapple to dapple as this produces "extreme dapples" which are blind, deaf and often abnormal in other respects. Somewhat similar findings are recorded in Great Danes. Great Danes may be black, fawn, brindle, harlequin, or merled in colour, but "White" Danes also occur. The black, fawn, and brindle danes have normal eyes, but the harlequin and merled animals exhibit clinically minor anomalies which closely resemble those seen in the dappled dachshund. Harlequin and merled animals do not breed true; inbreeding within each of these produces litters containing "white" puppies: these are seldom reared as they are known frequently to become blind and deaf.

This study is concerned with the pathological findings in an "extreme dapple" dachshund derived from a mating of dapple to dapple. A rather less detailed account is given of the eyes of some normal and abnormal newborn Great Dane puppies.

"EXTREME DAPPLE" DACHSHUND

Clinical Features †

The animal, aged 3 months, was examined on the day it was killed.

The sclera had a bluish hue and episcleral vessels were present. A pair ran parallel to the limbus and 5 mm. from it on the temporal side of the right eye. In the other eye a group of 3 lay on the upper side of the eye and entered the globe 5 mm. from the limbus. None of these vessels was so prominent as those seen previously in dappled dachshunds.

The cornea appeared small because of limbal encroachment by the sclera and ovoid because this encroachment was most marked superiorly.

*Working under a grant by the Medical Research Council from the Alexander Piggot Wernher Memorial Trust.

† *I am indebted to Mr. J. B. Davey for this account.*

The iris was thin and partly translucent. Pigment was only present near the upper pupil margin, this was more marked in the left eye. Elsewhere the colour of the iris was white tinged with blue. The pupil margin was ragged and in the left eye there was a persistent pupillary membrane stretching from 2 o'clock to 10 o'clock.

Both the lenses had subluxated and appeared as large floating opacities. The anterior chamber was deeper than normal though this appearance of depth may have been exaggerated by encroachment of the sclera over the cornea. No iris tremor was seen.

The optic nerve head was white and the retinal vessels were difficult to differentiate from the choroidal vessel in the periphery of the fundus, though they appeared normal near the disc. No tepetum was present and the fundus background had an albinotic appearance.

There was a large coloboma-like defect on the temporal side of the disc in the right eye and one on each side in the left.

General Post-mortem Findings

The animal was killed by intra-peritoneal pentobarbitone. The eyes were removed immediately and fixed intact in acid Zenker. A general post-mortem examination of the animal revealed two minor developmental anomalies other than the ocular condition to be described. The upper 10 rings of the trachea were imperfectly separated from each other. The first 4 rings were fused into two plates and the next six into a single grooved plate. In the heart there was a small residual foramen ovale which probably had no functional effects.

The Excised Eyes

Macroscopic appearances

Before removal of the eyes the upper edge of the cornea was marked with a red hot needle. After fixation the eyes were divided through a horizontal plane passing just above the disc.

Right eye. The right eye was elongated in its lateral axis due chiefly to ectasia of the sclera of the temporal half of the globe. The antero-posterior diameter was 15 mm. and the lateral 20 mm. The lens was dislocated and adherent to the posterior surface of the iris.

Left eye. The left eye measured 15 mm. antero-posteriorly and 18 mm. laterally. The lens was dislocated downwards. A short crescentic fold of retina running transversely over a small crater was located at the inferior pole.

The lenses were removed and the two halves of each eye embedded in paraffin wax. Sections of the lower halves were cut to a depth including the full thickness of the disc. The upper and lower blocks were then divided in half again at right angles to the original plane of section and more sections prepared from the nasal surfaces. All sections were stained with Ehrlich's haematoxylin and eosin and a few with van Gieson.

Histological features. Some histological features were common to both eyes. The corneoscleral angle was straightened out so that the curves of the cornea and sclera appeared continuous and of the same radius. The conjunctiva encroached on the area of the cornea. The ciliary muscle was less than half the size seen in a normal dog of comparable size, and pigment granules among its fibres were scanty. The iris had a stumpy triangular cross-section unlike the normal iris which has a truncat base. The posterior epithelial layer of the iris was lightly pigmented, and circular and radial muscle fibres were present in the stroma. The stroma itself was sparse and almost unpigmented. These features are illustrated in Fig. 1 which shows the ciliary region of the dachshund (a) and a Manchester terrier (b) of comparable size.

Right eye. In the immediate neighbourhood of the disc there was normal retina and pigment epithelium, except that the latter was unpigmented. Beneath the retina the choroid consisted of sparse stroma almost devoid of any pigment. Medially and laterally the retina became arcaded and tailed off into disorganized remnants merged into the ectatic sclera, but extended to the upper and lower poles as a normal or near normal structure. The disc showed a shallow depression deepest on its medial side. There was a wedge-shaped piece of retinal tissue beneath the medial wall of the depression. On the temporal side the retina did not appear to end sharply at the margin of the disc, scanty retinal elements were buried in the floor of the depression. The disc of the right eye is illustrated in Fig. 2.

Left eye. The retina and pigment epithelium were normal near the disc and extended upwards, but not laterally. The choroid had essentially the same appearance as in the right eye, and neither it nor the pigment epithelium contained pigment. There was a transverse fold in the retina at the lower pole with retinal rosettes in its angle. Beyond the fold disorganised retinal remnants lay directly on the sclera. The choroid appeared to end at the edge of the fold and actually lay above the rosettes in the angle. There was a small cyst in the sclera near the ora serrata which was lined by ciliary-like epithelium itself showing cystic changes. The disc showed a shallow depression deepest at the medial and inferior margins. The retina did not end sharply at the margins of the disc and there were numerous retinal elements, some organized into rosettes, submerged in the floor of the depression. All the features described are illustrated in Fig. 3. Between the temporal and superior poles and lying partially outside the sclera was a clearly distinguishable mass of retinal tissue. Rod and cone nuclei and ganglion cells were all easily identified. An artery of medium size in oblique section was noted in the sclera closely related to the inner side of the mass, but its source was not traced.

GREAT DANES

Nine newborn Great Dane puppies which had been discarded by a breeder were examined. The puppies were derived from three separate litters. Two were black with white underparts; one was merled; and the remaining six were white with one to three black or merled spots up to one inch in diameter. but it was impossible to determine which were "white" puppies with a few black spots, and which were "mismarked" harlequins. The eyes were fixed in acid-Zenker and sections cut through the median sagittal plane after removal of the lens. General post-mortem examinations were made with negative results.

Histological Findings

The eyes of the two black puppies were normal and corresponded in appearance with the description given by Parry (1953). The remaining seven puppies' eyes were all much less heavily pigmented than normal. Only in the merled puppy was there any pigment in the choroid, iris stroma and pigment epithelium; in the six "white" puppies traces of pigment were confined to the posterior epithelial layer of the iris and the ciliary body. No abnormality of the retina was apparent at this early stage of differentiation. In none was there a normal tapetum lucidum.

The merled puppy and the six "white" puppies appeared to fall into two groups: in four—the merle and three whites—the choroid was deficient in pigment and lacked a tapetum, but showed a vascular arrangement resembling the normal. The merled puppy had a definite tapetum, but the cells were fewer in number and not sharply separated from the surrounding choroid; one of the "white" puppies had even scantier rudiments of a tapetum, but no evidence was present in any of the others, although, since serial sections were not cut, the presence of rudiments cannot be excluded.

In the second group of three "white" puppies, not only was there no tapetum and no choroidal pigment, but the choroid itself appeared much thinner and less vascular than normal—and was similar to the choroid surviving in the dachshund eyes.

Fig. 4 illustrates the appearance of the choroid and tapetum of the normal black puppy (a) compared with the rudimentary tapetum of the merled puppy (b) and the grossly defective choroid of the second group (c).

In all the abnormal puppies except the merled, the iris and ciliary body appeared hypoplastic compared with the normal. The iris was flat or stumpy and triangular in cross-section, but invariably contained both radial and circular muscle fibres. It is, however, difficult in an undeveloped eye to decide exactly how serious these deviations are, but their occurrence is of interest in relation to the gross hypoplasia of these structures in the dachshund.

DISCUSSION

Microphthalmos is associated with numerous defects, but none of the descriptions given in the literature correspond closely with the findings recorded here. Schleich (1893) and Zimmerman (1897) described anomalies in various dogs consisting of microphthalmos, reduction in corneal area and circumscribed scleral staphylomata; reduction in the extent of the tapetum lucidum was also noted in these eyes. The eye described by Parsons and Coats (1906) which was adherent to an orbital encephalocoele also had somewhat similar defects.

Three separate processes of hypoplasia, aberrant development and secondary degeneration have contributed to the pathological picture seen in the dachshund eyes. The most important of these is a general hypoplasia. The eyes were small, the entire uveal tract was greatly underdeveloped and there were other immature features such as the absence of the normal corneo-scleral angle and encroachment of the sclera on the cornea. Pigmentation was much less than normal. Small amounts of pigment were confined to the iris stroma, choroid and ciliary body, but the posterior epithelial layer of the iris contained a near normal quantity. In contrast are those features resulting from aberrant development as exemplified by the minor anomalies, probably colobomatous in nature, seen in both discs; and in the left eye by the cyst of oral epithelium and associated retinal fold and the small mass of retina displaced into the sclera. Unlike the surviving tissue of the uveal tract, such retina as remains is essentially normal in structure. Furthermore, the circular and radial muscle fibres of the iris, which are neuroectodermal in origin, were present despite the extreme hypoplasia of the iris. Secondary degenerative changes account for the coloboma-like areas which have most probably developed in areas of grossly imperfect differentiation of the choroid where the retina has become completely disorganized and finally in the right eye ectasia of the sclera has followed.

It is impossible to estimate at what period of embryonic development the eyes were first retarded, but aberrant development must have occurred during the completion of closure of the foetal fissure since the lesions of the disc are likely to be due to anomalous closure of the central end of the fissure and the cyst of oral epithelium the peripheral end.

In the Great Danes there was no evidence of aberrant development, but, since the aberrant development seems to be a somewhat subsidiary feature almost confined to the left eye in the dachshund, this does not constitute a major difference. Hypoplasia cannot easily be assessed with much precision in an immature organ and in making comparisons allowances must be made for this and also for the fact that it is too early for degenerative changes to be seen. Evidence of hypoplasia was nevertheless present in all the abnormal

puppies. In the first group the choroid was abnormal chiefly in its lack of a normal tapetum and deficient pigmentation, and the puppies of this group fit in well with the clinical observation of minor anomalies of the tapetum and iris in heterozygous animals (Sorsby and Davey 1954). In the second group the lesion was much more severe and the choroid closely resembled the choroid surviving in the dachshund; it therefore seems reasonable to conclude that these are homozygous animals which would have been obviously microphthalmic if they had reached maturity. Since the tapetum is the last part of the uveal tract to become fully differentiated (Parry 1953), and also probably the most specialized, it is not surprising that the most consistent minor defect in differentiation of these tissues seen in the heterozygotes should be confined to the tapetum.

CONCLUSIONS

A clinical and histological account is given of the eyes of a "white" dachshund—an animal homozygous for dappling. There was microphthalmos and general hypoplasia of the uveal tract associated with minimal developmental aberrations which had probably arisen during closure of the foetal cleft.

Some newborn Great Dane puppies, which were not readily distinguishable into types, including some apparently normal, some heterozygous for the harlequin pattern and some homozygous for it (these latter corresponding to the "white" dachshund), were also studied. A tapetum lucidum was present in the normal puppies, but lacking or hypoplastic in the homozygotes while in the heterozygotes the choroid itself was hypoplastic.

ACKNOWLEDGMENTS

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LEGENDS FOR FIGURES

Fig. 1. (a) "Extreme dapple" dachshund. Iris and ciliary body of left eye in the mid-ventral region showing the extreme hypoplasia of these structures.

(b) Manchester Terrier. Ciliary region of left eye on the nasal side showing the normal appearance of this region in the dog for comparison with (a).
Both sections stained with haematoxylin and eosin. Magnification × 100.

Fig. 2. "Extreme dapple" dachshund. Section through disc of right eye to show small coloboma of disc. Stained with haematoxylin and eosin. Magnification × 30.

Fig. 3. "Extreme dapple" dachshund. Mid-ventral region of left eye showing the edge of the transverse fold and part of the oral cyst.
Stained with haematoxylin and eosin. Magnification × 30.

Fig. 4. Great Dane puppies.

(a) Normal choroid and tapetum.

(b) Choroid showing an imperfectly developed tapetum.

(c) Grossly defective choroid with no evidence of tapetum.
All sections stained with haematoxylin and eosin. Magnification X 300.

Fig. 1.

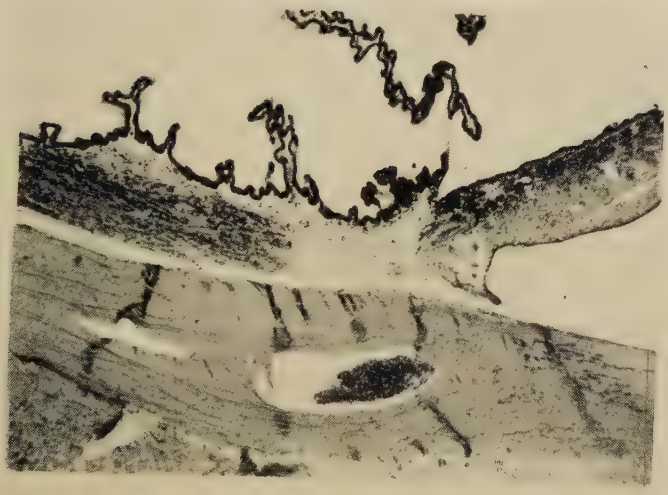

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Fig. 2.

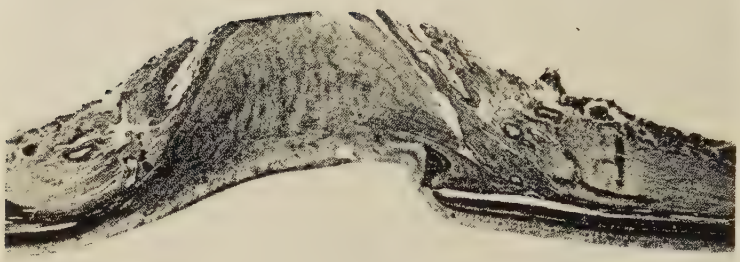


Fig. 3.

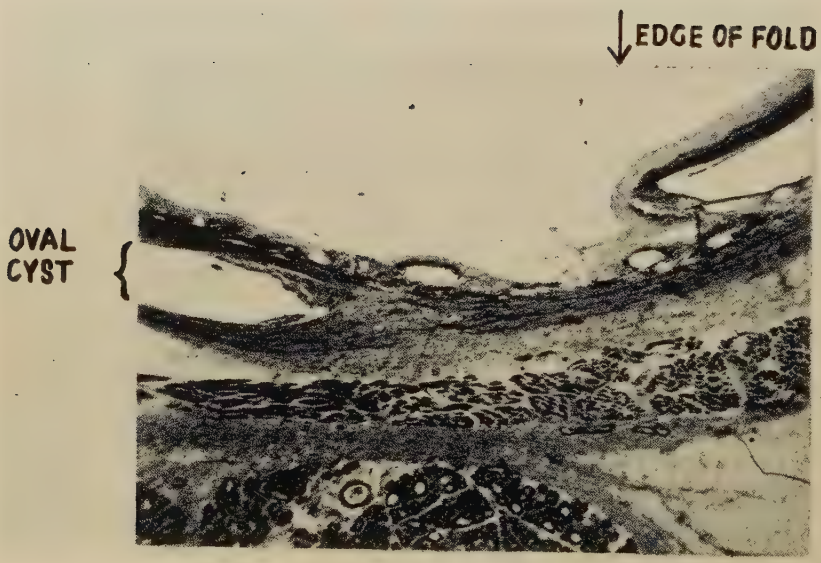
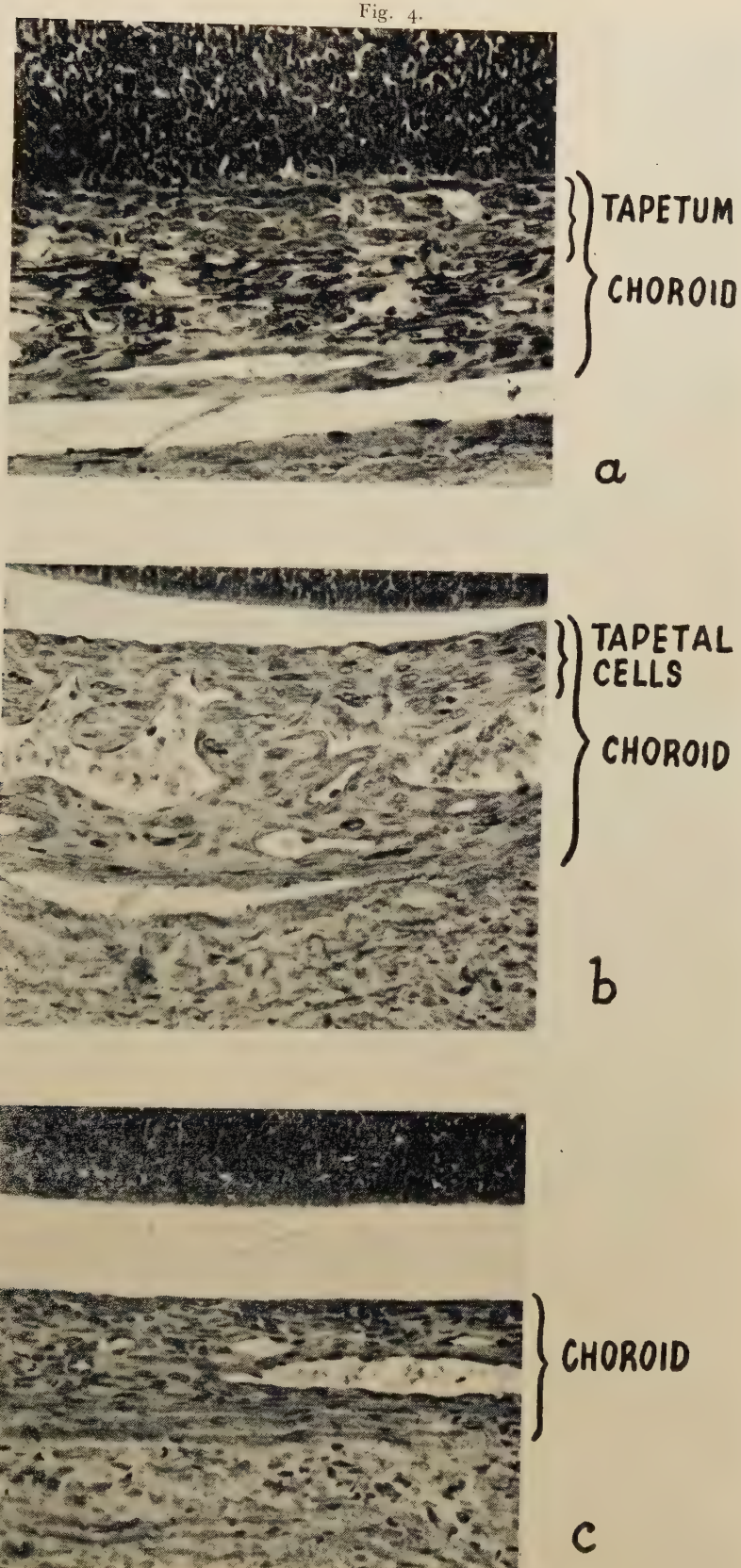


Fig. 4.



SKIN HYPERSENSITIVITY IN CATTLE INFESTED WITH *FASCIOLA HEPATICA**

By

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INTRODUCTION

Sievers and Oyarzun (1932) found that sheep infested with *Fasciola hepatica* developed an allergic response to both cutaneous scarification and intradermal inoculation of extracts of the parasite. This observation was confirmed and extended to other domestic animals by Wagner (1935), Aygun and Baskaya (1939), Szaflarski (1950) and Sobiech (1951). It was found by other workers that humans infested with *F. hepatica* reacted in the same way (Rukawina, 1935; Morenas, 1943 and 1944 and Mazzotti, 1948, 1952). Such intradermal tests have been used in the diagnosis of fascioliasis in both man and animals and have been regarded as satisfactory. Their success has been judged however, in many instances, on the presence of *F. hepatica* ova in the faeces, a criterion which is open to uncertainty.

In this investigation the results of the skin tests were related to the postmortem evidence of infestation by *F. hepatica*

METHODS

Antigens. The method of Trawinski (1935) was employed as follows:

Mature *F. hepatica*, collected from the bile ducts of slaughtered cattle and sheep, were washed in sterile saline, incised to allow the caecal contents to escape and after further washings the remaining portions were dried at 37°C. for four days. Subsequently the dried residue was reduced to a fine powder in a sterile mortar and stored at −10°C. until required. The powder was extracted at 4°C. for ten days, one gramme of the pulverised material being extracted in 100 ml. of saline, the mixture being shaken at frequent intervals. After extraction the suspension was filtered and centrifuged, the resulting clear solution being sterilised at 56°C. for 45 minutes, centrifuged again and the supernatant fluid was placed, in one millilitres aliquots, into small bottles fitted with a rubber stopper and these were stored at −10°C.

A control solution consisting of a one per cent solution of peptone was employed to detect non-specific skin sensitivity. In some animals an extract of Echinococcus cyst was used to determine whether cross specificity existed between this parasite and *F. hepatica.*

Injections. Dental syringes with a graduated plunger shaft were used for injection purposes. The injections were made intradermally in 0.1 ml. amounts at the selected sites, these being at the middle third of the neck on a line parallel to the spine of the scapula. The sites, four inches apart,

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were clipped free of hair and the thickness of the fold of skin measured by calipers and recorded. The injections of the antigenic and control solutions were then made. The subsequent reactions were observed after four hours and the thickness of the skin folds was again measured and recorded.

Post-mortem examinations. All animals were numbered before intradermal injections were made. Slaughter was carried out usually within twenty-four hours of the test being performed. Following slaughter, the viscera and carcase of each animal were examined for *F. hepatica.* Where no macroscopic evidence of *F. hepatica* infestation was found the contents of the gall bladder were collected, centrifuged and any deposit examined for trematode ova. The presence of other somatic helminths was recorded. A note was made of any bacterial disease, especially tuberculosis, which was present in the carcase or its viscera and the state of the nutrition.

RESULTS

Character of Reactions

After injection of the antigen material the site was marked by a small pea-like swelling about four mm. in diameter. In animals which reacted to the intradermal test this initial swelling increased in size and after an hour consisted of a flat elevated wheal which was surrounded by a varying degree of oedematous infiltration. This infiltration reached its maximum in four hours and was maintained for a further two to four hours. After twenty-four hours the reaction had resolved, the site being marked only by a slight thickness of the skin. In white-skinned animals the reaction was surrounded by a zone of erythema.

The skin reaction was characterised by marked oedema and perivascular infiltration of polymorphonuclear leucocytes, which appeared as early as twenty minutes after injection of the *F. hepatica* extract. The majority of the leucocytes were eosinophils.

Reactions to the peptone solution were very limited and without wheal formation. In some animals, however, there was a slight infiltration producing an increased skin thickness within four hours of one to two mm. Some of the animals which reacted strongly to the *F. hepatica* extract (30 mm. to 40 mm.) showed an oedematous reaction to the peptone but this was in no way comparable with that evoked by the specific antigen.

It was possible to test 164 cattle infested with *F. hepatica* and the results are presented in Table 1. It will be observed that 90.25 per cent showed a skin reaction of 6 mm. or more. Of the remaining sixteen animals, twelve (75 per cent) developed a reaction of 5 mm. and four showed increased measurements of from 1 mm. to 4 mm. The latter were emaciated and suffering from intercurrent disease, three being affected with suppurative cholecystitis and one with suppurative mastitis. It is possible that these animals were examples of desensitisation induced by cachectic and suppurative conditions as instanced by Botteri (1922), Spengler (1923) and Deusch (1925) who observed similar loss of skin sensitivity following suppuration

TABLE 1

FREQUENCY DISTRIBUTION OF REACTIONS IN INFESTED ANIMALS

Age Group	*Number of Animals*	*Number showing the following increases in skin thickness (mm.)*													
		1-2	3	4	5	6	7	8	9	10	11	12	13	14	15 or *over*
1–2 years ..	6	—	—	— (2)	—	1	1	—	—	—	1	2	—	—	1
2–3 years ..	47	— (3)	—	—	2	9	5	4	5	3	4	1	1	3	10
3–4 years ..	61	— (3)	—	—	7 (1)	14	3 (1)	5	7	4	7	2	2	1	9
4–5 years ..	22	—	—	1	1	3	—	4	2	1	—	1	—	1	8
5–6 years ..	13	—	— (2)	— (1)	1	5	1	1*	2	—	1	—	1	—	1
7 years and over	15	2	—	1	1	3	—	5	1	—	—	—	2	—	—
Total ..	164	2 (3)	— (5)	2 (3)	12 (1)	35	10 (1)	19	17	8	13	6	6	5	29
Percentage	100	9·75				90.25									

The figures in parenthesis indicate the increase in skin thickness in animals injected with 1–100 hydatid extract.
* Animal infested with *Cysticercus tenuicollis*.

of hydatid cysts. In the majority of animals the reaction was proportional to the degree of infestation and this correlation was most marked in animals between two and four years of age.

Reactions to an extract of hydatid cysts were of an insignificant nature and on autopsy no animal was found to be infested with echinococcal cysts. One was infested with *Cysticercus tenuicollis* but did not react to the hydatid extract.

Forty-eight of the animals tested were not infested with *F. hepatica:* the results are presented in Table 2. It will be observed that 83.33 per cent of the skin reactions were below the arbitrary level of 6mm. which was adopted in infested animals. Marked increases in skin thickness of 15 to 40 mm. such as were evident in twenty-nine of the infested animals were not observed in non-infested animals.

An attempt was made to prepare a more purified antigen in order to produce a clearer definition between affected and non-affected animals. Purified protein obtained by trichloracetic acid precipitation was tried but proved unserviceable for intradermal tests.

TABLE 2

FREQUENCY DISTRIBUTION OF REACTIONS IN NON-INFESTED ANIMALS

Age Group	*Number of animals*	*Number showing the following increases in skin thickness in millimetres*								
		1–2	3	4	5	6	7	8	9	10 or over
1–2 yrs.	7	2	3	1	1	—	—	—	—	—
2–3 yrs.	6	1	1	1	1	2	—	—	—	—
3–4 yrs.	15	4	1	7	1	2	—	—	—	—
4–5 yrs.	9	—	3	1	2	3	—	—	—	—
5–6 yrs.	6	—	2	2	2	—	—	—	—	—
7 yrs. & over	5	—	1	2	1	—	—	1	—	—
Total	48	7	11	14	8	7	—	1	—	—
Percentage	100	83.33				16.66				

Passive Sensitisation

Experiments to determine whether or not hypersensitivity to *F. hepatica* was due to circulating antibody were carried out using the Prausnitz-Küstner (1921) reaction. Serum was obtained from the heart blood of animals which had shown a marked reaction to the intradermal test or had failed to react. This serum was injected in one ml. quantities into the skin of the neck of non-reacting animals. Twenty-four hours later the prepared site was injected with 0.1 ml. of *F. hepatica* solution and the subsequent reaction was measured after four hours.

The sera of twenty animals were examined by this method. the results being presented in Table 3. Sixteen of these animals had originally shown a marked reaction to the *F. hepatica* material but only two of the sera produced passive sensitisation of normal bovine skin. Sera from non-sensitive animals did not cause any increased sensitivity on passive transfer to normal skin.

TABLE 3

Initial skin increase in donor animal to 0.1 ml. of saline extract (mm.)	*Increase in skin thickness at the prepared site after transfer of serum in recipient animal to 0.1 ml. of saline extract (mm.)*	*Initial increase in skin thickness to 0.1 ml. of saline extract in the recipient animal prior to passive transfer (mm.)*	*Increase in skin measurements as a result of passive transfer of sensitivity (mm.)*
10	3	3	—
4	4	3	1
10	5	4	1
4	4	4	—
6	5	5	—
11	8	5	3
10	5	5	—
11	4	4	—
10	6	4	2
2	3	3	3
14	14	6	8*
11	8	4	4
11	8	4	4
9	7	5	2
8	8	4	4
11	8	5	3
9	8	4	4
12	11	3	8*
2	4	4	—
14	7	4	3

*Animals in which passive transfer of sensitivity was demonstrated.

Desensitisation

To establish whether the reactions obtained with *F. hepatica* antigens were an expression of specific sensitisation, an attempt was made to effect desensitisation. To produce local skin desensitisation the same site was subjected to re-injection with 0.1 ml. of antigen solution three times at intervals of twenty-four hours. The reaction after each injection was measured four hours later. To induce generalised desensitisation three ml. of the *F. hepatica* solution were injected intradermally on one side of the neck and twenty-four hours later the usual intradermal test was carried out on the opposite side of the neck.

It was possible to test six cattle for local skin desensitisation the results being presented in Table 4. In all cases the reaction produced by the third injection of antigen was substantially smaller than the original reaction.

TABLE 4

FREQUENCY DISTRIBUTION OF THE INCREASE IN SKIN MEASUREMENT FOLLOWING THE RE-INJECTION OF THE INITIAL SITE WITH *F. HEPATICA* EXTRACT.

Initial increase (mm.)	*Increse after 2nd injection (mm.)*	*Increase after 3rd injection (mm.)*
20	13	5
6	4	3
19	8	8
16	9	7
23	14	8
8	6	3
Average Decrease	6.16	9.66

Table 5 shows that fifteen cattle were subjected to general desensitisation; eleven of these showed a varying degree of desensitisation, two were unaffected, whilst two showed an increase in skin sensitivity.

TABLE 5

FREQUENCY DISTRIBUTION OF THE INCREASE IN SKIN MEASUREMENTS (FOLLOWING THE INTRADERMAL INJECTION OF 3 ML. OF *hepatica* EXTRACT TO PRODUCE DESENSITISATION) TO 0.1 ML. OF *F. HEPATICA* EXTRACT (MM.)

Initial increase prior to desensitisation	*Increase after desensitisation*	*Decrease in reaction after desensitisation*	*Increase in reaction after desensitisation*
12	6	6	
18	9	9	
11	11	—	
8	8	—	
17	12	5	
24	18	6	
18	21	—	3
19	17	2	
11	9	2	
10	7	3	
9	3	6	
18	21	—	3
22	14	8	
19	13	6	
19	9	10	

DISCUSSION

The results of the intradermal tests indicate that hypersensitivity to *F. hepatica* can be demonstrated in the great majority of cattle infested with the parasite. In some animals sensitivity was very marked and in one animal was manifested by an increase in skin thickness of forty mm. following the injection of the antigen. The rapid response to the antigen, the nature and histological appearance suggest that the reaction is of an anaphylactic type. Like skin reactions to other helminth diseases, such as echinococcosis (Botteri, 1922; Tanturi, 1939), it was observed to be characterised by the appearance of a marked eosinophilia and oedema, which conforms with the anaphylactic response as described by Dienes

(1930), who observed a quickly developing oedema and an intense accumulation of leucocytes.

The reaction was found to be suppressed or inhibited by desensitisation of a local area of skin but general desensitisation could not be regularly produced by the intradermal injection of large quantities of antigen. The ability to induce desensitisation indicated that the reaction was a hypersensitive response and that *F. hepatica* was responsible for the production of the hypersensitive state.

Reactions observed with non-infested animals were generally slight and there were no very marked responses such as those seen in infested animals. In a number of non-infested animals (16.66 per cent) moderately increased reactions were observed. In these animals a previous minor or abortive infestation with *F. hepatica*, undetectable post mortem, may have rendered them hypersensitive.

Thirty-two cattle which were examined were infected with tuberculosis. It has been suggested by Harrell and Horne (1945), Harrell, Horne, Aikawa and Helsabeck (1947) that tuberculosis in humans increased their sensitivity to helminth antigens (*Trichinella spiralis*) and Kochnev (1950) has reported that cattle with pulmonary fascioliasis frequently reacted to the tuberculin test in the absence of tuberculosis infection. No evidence was found in this investigation to suggest that tuberculosis sensitised animals to the *F. hepatica* antigen.

SUMMARY

The results of this investigation confirm and extend the work of previous authors and establish that infestation with *F. hepatica* produces a hypersensitive state in the body.

Infestation with *F. hepatica* in cattle can be diagnosed by means of an intradermal test with approximately ninety per cent accuracy, the test being relatively specific.

Hypersensitivity could be induced passively by transfer of serum.

Desensitisation of a local skin area was produced but generalised desensitisation could not be effected to such a marked degree.

ACKNOWLEDGMENTS

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ANTIBIOTIC THERAPY IN STAPHYLOCOCCAL INFECTION OF THE BOVINE UDDER

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INTRODUCTION

It has been reported in the literature that therapeutic treatment of staphylococcal infection of the bovine udder, especially with penicillin, is not as successful as in the case of streptococcal infection. Most workers have found that repeated injections have brought about only a temporary reduction in the numbers of organisms secreted in the milk together with some improvement in the clinical condition of the udder. This apparent failure of penicillin to eliminate staphylococci is not easy to explain since strains of bovine staphylococci are reported to be as sensitive to its action in vitro as are streptococcal strains recovered from the udder.

Other antibiotics have more recently been used in this form of mastitis with varying results. Thus McCulloch, Kiser and Migaki (1949) reported cures in 85 per cent of infected quarters treated with 2 doses of 200 mg. aureomycin while Slanetz and Allen (1950) showed that as many as 96 per cent of chronically infected quarters could be freed from infection by injecting 3 similar doses and remained free for at least two months. Packer (1950) has demonstrated a reasonable response (i.e. in about 68 per cent of infected quarters) by using 2 injections of 200 mg. and Hulse (1951) has confirmed the therapeutic value of aureomycin in clinical cases of mastitis. On the other hand, Schalm (1950) failed to demonstrate a high rate of recovery in quarters treated with aureomycin but accounted for the lack of response by the fact that many quarters had received previous forms of treatment. Better results were reported later by Schalm and Woods (1952) by the combined use of penicillin and streptomycin. Thus, 70 per cent of lactating quarters and 73 per cent of dry quarters responded to large doses (1 million units of penicillin and 1 gm. dihydrostreptomycin) when infused four times at intervals of 24 hours.

The present study was conducted in order to investigate further the value of penicillin, aureomycin and streptomycin in staphylococcal infection of the udder. As a preliminary to this work, studies on the in vivo potency of these antibiotics against experimental infection in mice had demonstrated that they gave equal protection against moderate test doses (Edwards, 1952).

MATERIALS AND METHODS

Cows treated. Treatment was carried out in 37 naturally infected cows belonging to two dairy herds maintained at this Field Station. They were selected for treatment on the basis of two positive bacteri-

ological tests on milk samples carried out at the dairy. They were then removed to an isolation unit where they were submitted to a further series of six tests in a two week pre-treatment period.

Milking at the unit was carried out by a technique which was designed to limit the spread of infection as far as possible. This consisted in the use for each cow of an individual cluster which had been newly steam sterilised before each milking. Previous to milking, the udder was washed with a sterilised cloth soaked in hypochlorite solution (200 p.p.m. available chlorine) and, before applying the cluster, the milker dressed his hands with a cream containing 0.5 per cent 'Cetavlon'.

Examination of milk samples. Quarter samples were taken from each cow and 0.2 ml. of milk plated in ox-blood agar; the plates were incubated for 18 hours at 37°C. Typical colonies of staphylococci showing α or $\alpha\beta$ haemolysis were counted and one colony from each infected quarter was examined as follows :—

(a) The coagulase tube test, using both rabbit and sheep plasma, which was read after 2 hours incubation at 37°C. and after a further 18 hours at room temperature.

(b) The sensitivity of the strain to the antibiotic used in treatment was examined by the methods described by Edwards (1952). If infection was not eliminated by treatment, the strain was again examined to ascertain if it had developed increased resistance.

(c) The identification of bacteriophage type of the strain.

The reaction of milk samples was judged by the bromcresol-purple test and the cellular content by the Tromsdorff tube test. In the latter test, readings of 0.01 ml. per 10 ml. of milk, corresponding to a cell count of 1×10^6 per ml., were considered abnormal.

Treatment. The cows were assigned to four groups and each group was injected via the teat canal with one of the following antibiotics:—

(a) Aureomycin in a dose of 210 mg. or 420 mg. of the hydrochloride contained in 7.5 g. of ointment base. The injection was repeated after 4 days.

(b) Penicillin in a 100,000 unit dose of the calcium salt of penicillin G contained in a retaining base. Four doses were injected at two-day intervals.

(c) Streptomycin in a dose of 250 mg. of dihydrostreptomycin sulphate in a retaining base. Four doses at two-day intervals were injected.

(d) A combina ion of penicillin (100,000 units) and streptomycin (250 mg.) was injected using 4 doses at two-day intervals.

The number of injections and intervals chosen were made with a view to maintaining in the milk a continuous concentration of antibiotic for 8 days.

All quarters of infected cows were injected and the assessment of the efficiency of treatment was based on the absence of typical staphylococci on six tests at weekly intervals following treatment.

When quarters showed infection on two tests following the first course of treatment, the cows were re-injected. One cow resisting treatment in the first group was subsequently included in the last group.

RESULTS

Elimination of Infection and Effect on Milk

It will be seen from Table I that following one course of injections the result with all forms of treatment was approximately the same since about 70 per cent of quarters were freed from infection. A second course of treatment in cows resisting the first treatment increased the response to over 80 per cent except in the case of combined penicillin and streptomycin treatment. Thus, by combining the results of various treatments, infection was eliminated in 31 out of the 37 cows. In two cows which did not respond to two courses of treatment, further intramammary injection with the same antibiotic was not effective while one cow, which was included in both the aureomycin and penicillin-streptomycin trials without success, resisted 7 courses of treatment—a total of 22 separate injections. The three remaining cows failing intramammary treatment were injected intramuscularly with three daily injections each of 5 mega units of penicillin, aureomycin and streptomycin in succession but the staphylococci infecting one quarter of each cow persisted.

Daily milk yields and cell volume of the milk of the treated cows were recorded mainly to determine if injection of the various antibiotics produced any injurious effects. With regard to penicillin and streptomycin, no abnormal effects on the daily yield or on the character of the milk during treatment were observed, but the injection of aureomycin produced a slight temporary decrease in milk yield together with a clotting and discoloration of the milk, sometimes due to the presence of blood, for two to four days after treatment.

Character of Strains Isolated

Phage type. Thirty-five of the cows used in the experiment belonged to one herd from which the staphylococcal strains isolated always appeared to be of one type. They produced typically $\alpha\beta$ haemolytic colonies and coagulated both rabbit and sheep plasma within 2 hours incubation at 37°C. They were classified into the phage type 42D and conformed usually to the undiluted pattern 79/42D/54/77. (Groups I and III, Williams and Rippon, 1953).

The remaining two cows belonged to another herd where the prevailing staphylococcal infection was of two types and differed from that in the first herd. One of these cows was infected with a phage type 3A (Group II) and the other with a 52B type (Group III). Other characters of these two strains also served to differentiate them from the more common 42D type.

TABLE 1

ANTIBIOTIC THERAPY IN STAPHYLOCOCCAL INFECTION OF THE UDDER

Antibiotic	*Dosage*	*Interval between Injections*	*Before treatment*		*After one treatment*		*After two treatments*	
			Cows infected	*Quarters infected*	*Cows cured*	*Quarters cured*	*Cows cured*	*Quarters cured*
Aureomycin ..	2 x 210 mg.	4 days	11*	25	9	21 (84%)	10	22 (88%)
,, ..	2 x 420 mg.	4 ,,	5	12	3	9 (75%)	4	11 (92%)
Penicillin ..	4 x 100 mg.	2 ,,	9	23	6	16 (70%)	8	20 (87%)
Streptomycin ..	4 x 250 mg.	2 ,,	5	12	2	8 (67%)	3	10 (84%)
Penicillin .. + Streptomycin ..	4 x 100 mg. + 250 mg.	2 ,,	8	22	6	15 (68%)	6	15 (68%)

*One cow not responding to treatment with aureomycin was subsequently included in the last group.

Sensitivity to Antibiotics. Strains from the 16 cows treated with aureomycin were uniformly sensitive to 0.6 to 1.25 μg/ml. There was no evidence of the development of increased resistance in organisms recovered from the two cows failing treatment.

Strains from 8 of the 9 cows treated with penicillin were sensitive to 0.06 to 0. 125 μg/ml. but those isolated from the remaining cow were initially resistant to 5 to 10 μg/ml. of this antibiotic and belonging to the phage type 52B (Group III).

Strains from the five cows treated with streptomycin were sensitive to 1.25 μg/ml. and there was no alteration in the strains from the two cows resisting treatment.

Strains from the 8 cows treated with a combination of penicillin and streptomycin were uniformly sensitive to the two antibiotics. One cow included in this group had already received 4 courses (8 injections) of aureomycin without altering the sensitivity of the strain to this antibiotic. This treatment was followed by a further 3 courses (14 injections) of combined penicillin and streptomycin without inducing any change in the sensitivity of the infecting staphylococcus or in its phage type. The other cow resisting treatment in this group was infected with a phage type 3A strain which did not increase in resistance after 3 courses of treatment. The inclusion of these two highly resistant cows probably accounts for the slightly poorer result obtained with combined penicillin-streptomycin therapy.

DISCUSSION

The 37 cows used in this investigation were secreting milk which was visibly normal although they were harbouring pathogenic staphylococci in one or more quarters. But, further inspection showed that of the 94 infected quarters, the milk of 64 (68 per cent) contained an abnormal cell content. Since Plastridge, Williams and Weirether (1939) have also shown that 70 per cent of infected quarters secrete milk containing abnormal cell counts it may be regarded that infection with staphylococci is frequently responsible for the establishment of a chronic form of mastitis.

In this experiment an attempt has been made to compare and evaluate intra-mammary treatment with certain antibiotics in this type of affection. The results show that there was no evidence of the superiority of one antibiotic over another and that infection could be eliminated in 31 out of 37 cows. Further, it seemed clear that when two courses of injections failed to eliminate infection there was no advantage to be gained from continuing treatment. It would appear therefore that while antibiotic therapy against staphylococcal infection gives moderately good results there is reason to think that a greater number of cows resist treatment than is the case in streptococcal infection of the udder. The cause of such resistance might be attributable to differences either in the nature of the infecting

strains or in their antibiotic sensitivity. The present results however show that the failure to remove infection in 4 cows infected with staphylococci of the 42D phage type was not due to the development of increased resistance to the antibiotic employed even after continuous treatment. The two remaining cows failing penicillin treatment were infected with strains of different phage type—one of these being a sensitive 3A type (Group II) and the other a resistant 52B type (Group III). In view of the limited experience gained in this investigation, further work would be required to determine the relationship between the phage type of the infecting staphylococcal strain and the results obtained in therapy. In this connection it is interesting to note, however, that Williams and Rippon (1953) have recorded that a higher percentage of strains resistant to penicillin have been encountered among Group III strains recovered from human sources than in those belonging to other phage groups.

It has been shown by Schalm and Ormsbee (1949) that the response to penicillin is better in infected cows in their first to third lactations than in those over this stage and that optimal results in potentially curable cases are obtained by the first treatment. They also suggest, as a tenable explanation for the failure to cure *Staph. pyogenes* in long-standing infections, the localisation of staphylococci within the udder tissue. The present results tend to support these findings since four of the six resistant cows had had five or more lactations. It may be concluded, therefore, that antibiotic therapy may be usefully employed for the elimination of staphylococcal infection of the udder in young cows but that long-standing cases do not respond to treatment.

SUMMARY

A comparison was made of certain antibiotics injected via the teat canal in the treatment of staphylococcal infection of the udder of 37 lactating cows.

The results obtained showed that the value of aureomycin, penicillin, streptomycin, and a combination of the last two, was approximately the same and that about 80 per cent of infected quarters responded to one or two courses of treatment.

The six cows which were refractory to intramammary injection did not respond to further treatment which included the intramuscular administration of antibiotics to three cows.

It was found that failure to eliminate infection from the udder was not due to the development of increased resistance of the staphylococcal strain to the antibiotic employed but was related to the duration of infection.

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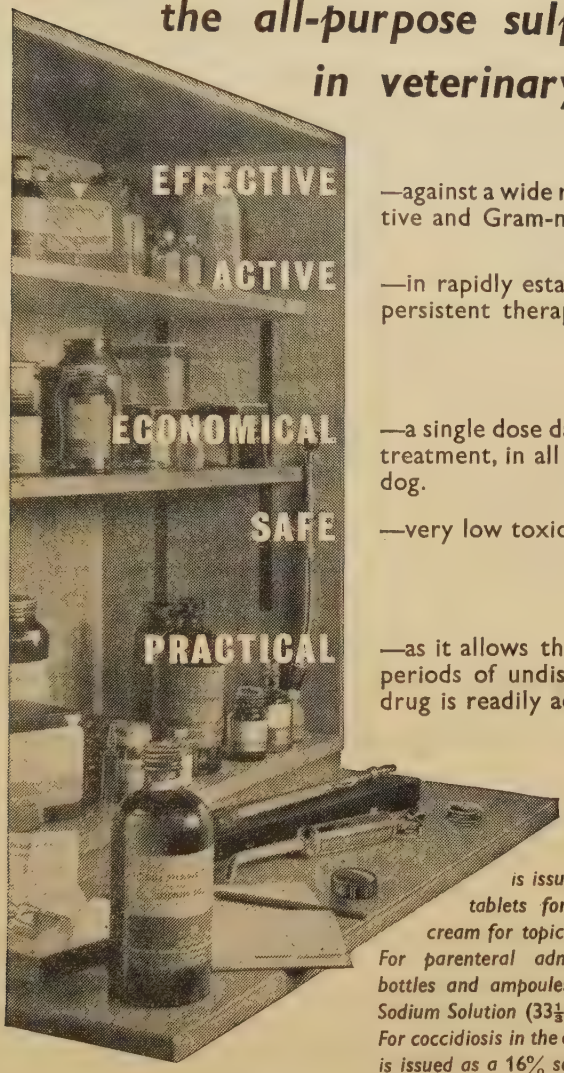

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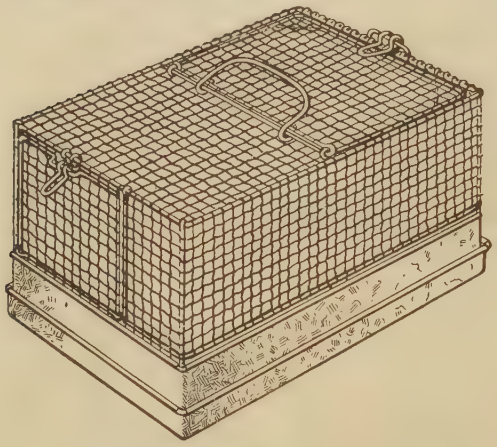

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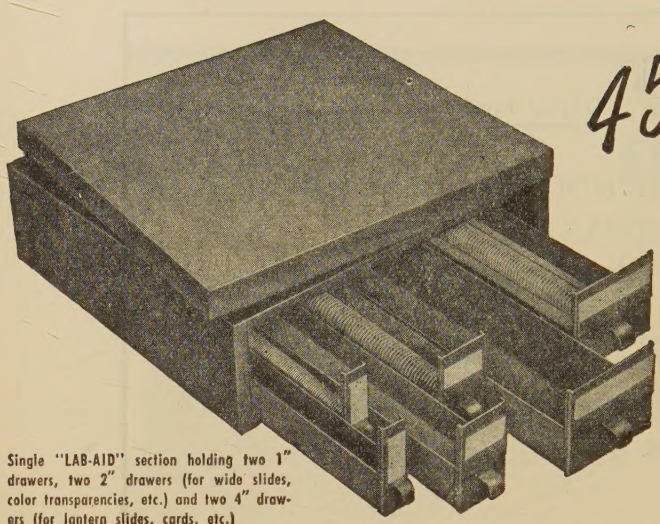

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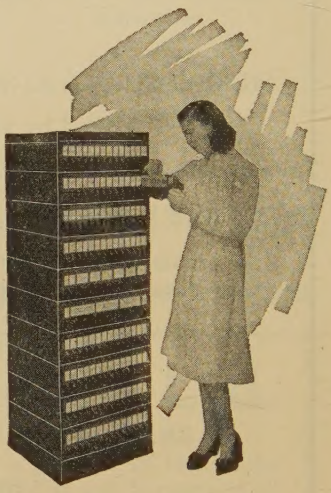

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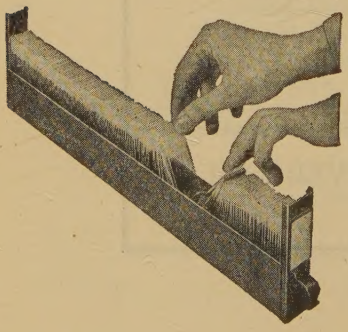

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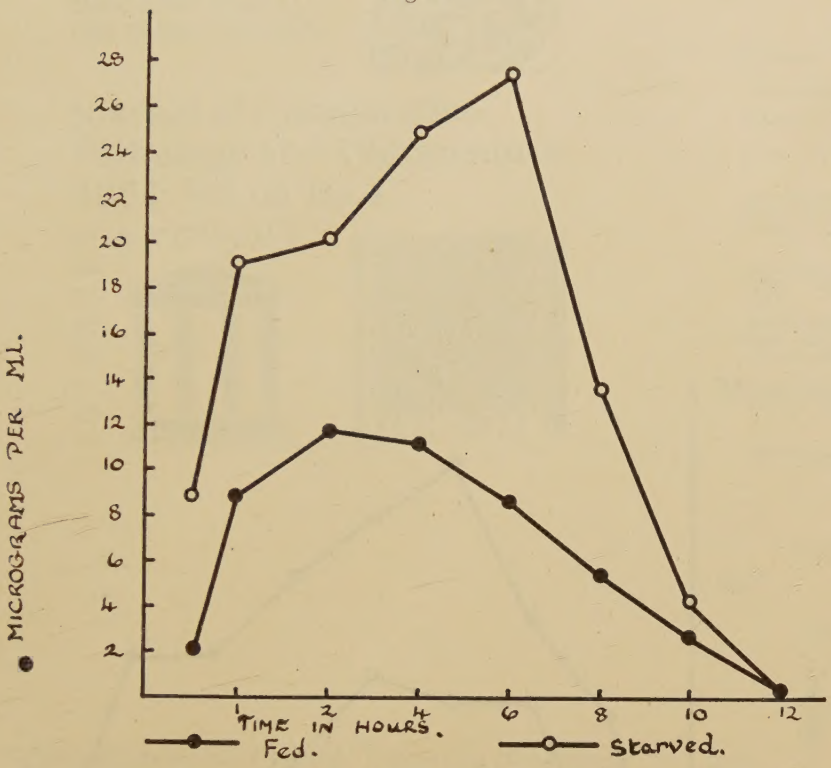

Fig. 2 Average serum levels of chloramphenicol in 8 starved and 8 well-fed chickens. Dosage 100 mg./kg. orally

Fig. 3

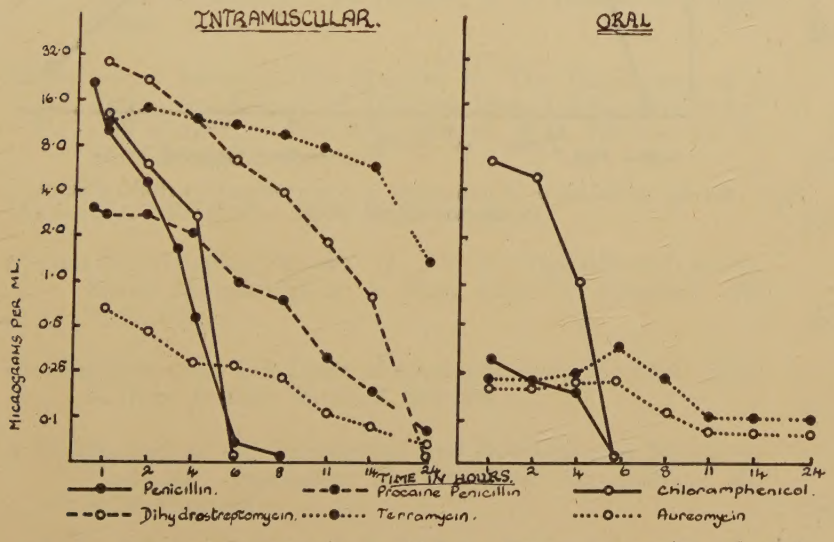

Fig. 3 Serum concentrations in chickens. Dosage 25 mg./kg. of each chemotherapeutic agent.

Fig. 1

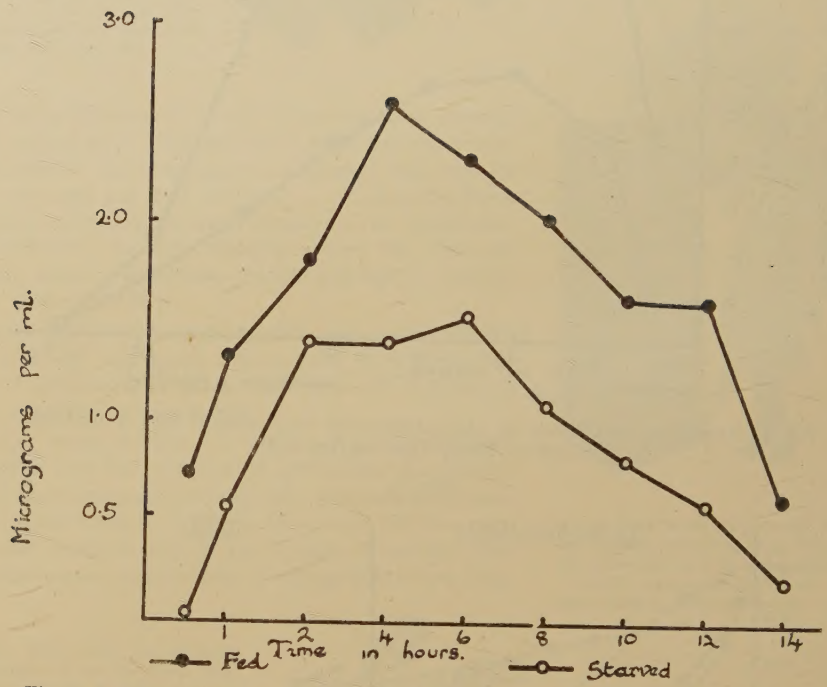

Fig. 1 Average serum levels of terramycin in 8 starved and 8 well-fed chickens. Dosage 100 mg/kg. orally.